

Enhancing the efficacy of TCR gene therapy

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ABSTRACT

TCR gene therapy allows redirection of the antigen specificity of T cells by the introduction of novel TCR α and β chains by retroviral transduction. These TCR gene modified T cells can be adoptively transferred to target defined tumour antigens. The majority of TCR gene therapy studies has focused on the adoptive transfer of CD8⁺ T cells but there is increasing recognition of a central role for CD4⁺ T cells in effective immunotherapy protocols. The use of CD4⁺ T cells has been limited by the lack of well defined class II restricted TCR and also because the majority of tumours don't express class II MHC. As a result research has focused on introducing class I restricted TCR into CD4⁺ T cells. Initial work has demonstrated that class I restricted CD4⁺ T cells often have reduced functional avidity compared to the parental CD8⁺ T cell. In particular, CD4⁺ T cells transduced with CD8 dependent TCRs are often of much lower functional avidity when introduced in the absence of a CD8 co-receptor. In order to improve the functional avidity of class I restricted CD4⁺ T cells, murine CD4⁺ T cells were co-transduced with F5 TCR (specific for influenza peptide, NP, in the context of H2-K^b) and additional CD3 molecules. The amount of CD3 within a cell is rate limiting for the expression of introduced TCR and thus when cells are transduced with additional CD3 it removes this rate limiting step and thus enhances the surface expression of the TCR. TCR surface expression is one of the key determinants of T cell functional avidity. CD4⁺ T cells co-transduced with F5-TCR and CD3 had increased surface expression of F5-TCR and increased pentamer binding. This translated in vitro into increased functional avidity

compared to CD4⁺ T cells transduced with F5-TCR only. When adoptively transferred in vivo into irradiated tumour bearing syngeneic recipients, F5-TCR + CD3 CD4⁺ T cells had greater expansion and persistence and trafficked to the tumour site at higher and faster rates than F5-TCR only CD4⁺ T cells. In addition, F5-CD3 CD4⁺ T cells demonstrated superior control of tumour growth. Unexpectedly mice that received adoptive transfer of F5-TCR + CD3 CD4⁺ T cells developed marked lethal toxicity. Further experiments to try to determine the nature of this toxicity suggest a multifactorial cause including mispairing of the introduced TCR α and β chains with the endogenous TCR and development of autoreactive T cells in the presence of additional CD3 mediated either by upregulation of the introduced TCR or the endogenous TCR.

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CHAPTER ONE – INTRODUCTION

1.1 INTRODUCTION

Adoptive Immunotherapy involves the active transfer of naturally occurring or gene modified T cells to target tumour or viral antigens within a recipient. Autologous tumour infiltrating lymphocytes (TILs) can be isolated from individual patients and expanded ex vivo prior to adoptive transfer. Another strategy to produce tumour specific T cells for adoptive transfer is to use retroviral transduction to introduce a tumour antigen specific TCR into polyclonal T cells thus redirecting their specificity. This was first demonstrated by Clay et al who transduced human T cells with a TCR that was specific for a melanoma antigen in the context of HLA-A2 (1). TCR gene transfer generates functional T cells that have the same specificity as the parental clone. TCR transduced T cells secrete cytokines and proliferate in response to specific antigen and can lyse antigen positive targets in vitro (2) (3). In vivo mouse models have also demonstrated that tumour specific TCR gene modified T cells can provide tumour protection (4) (5) (6).

The first clinical trial of TCR gene modified T cells in humans was used to treat patients with metastatic melanoma. Autologous T cells were transduced with a TCR specific for the melanoma antigen, MART-1 presented by HLA-A2. Transduced T cells were adoptively transferred to patients following non-myeloablative conditioning chemotherapy. Objective responses were seen in 2/15 patients (13%). Both responding patients had long-term disease control.

(7). A follow up trial used a TCR that targeted the same MART-1 epitope but with higher pMHC binding affinity and this led to anti tumour responses in 6/20 (30%) of patients treated (8). TCR transduced T cells that target the cancer testis antigen, NYESO1, have also been used to treat patients with metastatic melanoma or advanced synovial cell carcinoma where response rates of 45% and 67% respectively were observed (9).

Superior responses (up to 72%) are still seen following adoptive transfer of TILs compared to TCR transduced T cells, particularly in patients with metastatic melanoma (10). However, TCR transduced T cells have many advantages over TIL. This technique can be used to generate tumour specific T cells expressing TCR that are absent from the normal T cell repertoire due to central tolerance mechanisms and can also be used to generate high avidity tumour specific T cells. For the majority of malignancies it is not possible to isolate TIL in sufficient number. TCR transduction can be used to generate large numbers of tumour specific T cells by transduction of a patients autologous lymphocytes and thus can vastly increase the number of types of tumours that could potentially be treated by adoptive transfer of TCR transduced T cells. Initial studies of TCR gene therapy have focused on the adoptive transfer of CD8+ cytotoxic T cells but there is increasing use of CD4+ T cells in adoptive transfer protocols and increasing recognition of an important role for tumour specific CD4+ T cells in tumour eradication. However development of TCR transduced CD4+ T cells has not happened at the same pace as that of TCR transduced CD8+ T cells. One of the main reasons for this is that there is a lack of well-defined MHC class II restricted

tumour epitopes and thus fewer tumour specific class II restricted TCRs have been isolated for use in gene transfer experiments. The transfer of Class I restricted TCR can be used to generate class I restricted CD4+ T cells and has been shown to produce functional antigen specific CD4+ T cells. However the majority of class I restricted TCRs are CD8 co-receptor dependent and thus CD4+ T cells expressing class I restricted TCR tend to be of lower avidity than CD8+ T cells expressing the identical TCR. In addition, the full range of CD4+ T cell functions may be absent in the absence of a functional co-receptor interaction.

This project has focused on a strategy to try to improve the functional avidity of a class I restricted TCR transduced CD4+ T cell. Functional avidity is dependent on both the affinity of the TCR and also the density of TCR expression. The amount of transduced TCR that is expressed on a T cell surface is limited by the amount of CD3 within the T cell. The transduced TCR and the endogenous TCR must compete for binding to the endogenous CD3 molecules and thus it has been hypothesized that by transducing T cells with TCR plus additional CD3, an excess of CD3 can be provided and the competition for binding to CD3 reduced. This should result in higher surface expression of the transduced TCR thus leading to enhanced functional avidity of the class I restricted CD4+ T cell. This strategy of co-transfer of TCR and CD3 has already been explored in CD8+ T cells and has been shown to increase the in vitro functional avidity of T cells co-transduced with TCR and CD3 and this leads to superior tumour protection following adoptive transfer in vivo (5).

1.2 THE IMMUNE RESPONSE TO TUMOURS

1.2.1 Immune surveillance of cancer

The immune system has a key role in controlling the development and growth of tumours in vivo. However, tumours still develop despite the presence of an intact immune response. In a process termed cancer immune surveillance, the immune system detects transformed cells that have escaped from the cells' intrinsic tumour suppressor mechanisms and acts to eradicate malignant cells. This process is dynamic, and the behaviour of the tumour is shaped by the immune response directed against it and vice versa.

There is strong supporting evidence in both murine and human models for the presence of cancer immune surveillance. Mice that have a homozygous deletion of RAG2 (and thus lack T, B and NK cells) have an increased incidence of naturally occurring and chemically induced tumours than wild type mice (11). An increased frequency of tumours is also demonstrated in mice lacking other immune mediators such as IFN- γ , IFN- γ receptor or perforin (12) (13) (14). In addition, humans with either congenital or acquired immunodeficiency have an increased frequency of malignancy, especially virally driven tumours (15).

To assess whether the immune system could shape the immunogenicity of tumours, chemically induced sarcomas derived from immunocompetent wild

type mice and from immunodeficient RAG -/- mice were used in tumour transplantation experiments. When injected into RAG-/- recipients, tumours generated in both RAG-/- mice or wild type mice grew progressively and with similar kinetics. Similarly, tumours generated from wild type mice grew progressively when injected into immunocompetent mice. However 40% of tumours derived from carcinogen treated RAG-/- mice were rejected after transplantation into immunocompetent wild type mice even following injection of a very high tumour cell burden. This would suggest that tumours generated within mice that were immunodeficient were much more immunogenic than those derived from immunocompetent hosts. An intact immune system acts to edit and shape growing tumours, which as a result become less immunogenic and can evade and outgrow the immune response (11).

Schreiber et al proposed the theory of cancer immune editing which demonstrates how the immune response affects malignant cells in vivo (16). This theory divides cancer immune editing into 3 phases, elimination, equilibrium and escape. In the elimination phase, the immune system actively surveys to detect and destroy malignant cells as they arise. Numerous effector cells and pathways are important for suppression of tumour development. However with time this progresses to a state of dynamic equilibrium where the immune system acts to contain the tumour growth but does not fully eliminate the malignant cells. During the equilibrium phase tumour cells start to acquire gene mutations or changes in gene expression as a result of selection pressure exerted by the immune response, which leads to resistance to recognition and/or elimination. This then progresses to

the escape phase when the immune system fails to control these surviving tumour cell variants, which have reduced immunogenicity, resulting in uncontrolled proliferation.

Tumour cells have developed multiple methods to escape and evade recognition by the immune system. In simple terms, tumour cells can evade immune destruction either as a result of lack of recognition, resistance to cytotoxic mechanisms or by the induction of tolerance. Mutations may lead to loss of tumour antigens and development of antigen loss variants that are not recognized by T cells (17). In addition, tumour cells can acquire defects in antigen presenting and processing pathways including loss or down regulation of Class I MHC, β 2microglobulin, TAP, tapasin and proteasome components (18) (19) (20) (21). Other described mechanisms initiated within tumour cells to evade immune mediated killing are up regulation of anti-apoptotic molecules such as FLIP or Bcl-xl (22) (23). Tumour cells may resist killing via death receptors through the expression of mutated forms of TRAIL or FAS ligand (24) (25). Absence or abnormal function of components of the IFN- γ receptor signaling pathway leads to tumour cells becoming unresponsive to the effects of IFN- γ .

The tumour microenvironment itself is often suppressive with secretion of immune regulating factors that can prevent T cell infiltration or promote T cell exhaustion. Tumour cells promote the formation of an immunosuppressive environment by production of immunosuppressive cytokines such as TGF- β or IL-10 (26). Overproduction of Indoleamine 2,3 dioxygenase by tumour

cells or by dendritic cells (DCs) in the tumour microenvironment can lead to suppression or anergy of tumour reactive T cells and activation of T regulatory cells (27). DCs in tumour draining lymph nodes are often incompletely activated which may favour the induction of tolerance rather than of priming (28).

1.2.2 Processing and presentation of tumour antigens.

T cells recognize peptide in a complex with MHC molecules. CD8⁺ T cells recognize peptide presented by Class I MHC and CD4⁺ T cells recognize peptide presented by class II MHC. Class I MHC is composed of a polymorphic heavy chain bound to β 2-microglobulin whilst Class II MHC is composed of polymorphic α and β chains. Class I MHC is expressed on all nucleated cells allowing CD8⁺ T cells to detect infected or malignant cells. The expression of Class II MHC is restricted mainly to B cells, macrophages and DCs, which act as professional antigen presenting cells (APCs). Class II MHC expression can also be induced by IFN- γ on non-professional APCs such as fibroblasts and endothelial cells (29) or on tumour cells. Non-professional APCs however express variable levels of co-stimulatory molecules and thus have an inconsistent ability to process and present antigen. The transcription of MHC class II genes is controlled by the MHC class II trans activator (CIITA) that ensures tissue specific expression of class II MHC (30) (31).

Both the class I and class II MHC molecules fold to form a peptide-binding groove on their distal surface. Along the length of the groove are pockets into which key anchor residues of the peptide bind. The interactions between the anchor residues of the peptide and the pocket of the groove determine the specificity of peptide binding. Additional stability is provided by hydrogen bonds between conserved non-polymorphic amino acids within the MHC molecules. Within the class I MHC, the conserved hydrogen bonds are at either end of the peptide binding groove which limits the binding of peptides to those that are 8-9 amino acids in length only (32). In contrast, the peptide-binding groove of class II MHC molecules is “open” so theoretically peptides of any length can be bound. The polymorphic nature of the class I and class II MHC proteins generates a vast number of different peptide binding grooves (33) (34).

Peptides that bind to class I MHC and stimulate CD8⁺ T cell responses are predominantly derived from endogenous intracellular antigens whilst peptides that bind to class II MHC and stimulate CD4⁺ T cell responses are predominantly derived from exogenous antigens. However, exogenous antigens can also be presented by class I MHC in a process called cross presentation. In addition, autophagy allows endogenous antigens from the cytosol to enter the phagosomal network where they are be presented by class II MHC (35).

In order to become competent effector cells, T cells must be primed with specific antigen presented by professional APCs, which express in addition to

MHC class I or II, high levels of co-stimulatory molecules and relevant adhesion molecules. The most efficient APC is the DC, which are also the main cross presenting APC (36). Lymphoid organ resident CD8⁺ DCs are the most efficient at cross presentation particularly in steady state but in inflammatory conditions other DC subsets and macrophages also develop the ability to cross present (37).

DCs have a unique ability to acquire and present peripheral tissue antigens. They survey the peripheral tissues for the presence of infected or malignant cells. Following uptake of exogenous antigen they then migrate to draining lymph nodes, which are enriched in naïve T, cells which will scan DCs continuously looking for their cognate antigen-MHC (38). DCs express very high levels of class II MHC together with high levels of co-stimulatory molecules and are very efficient at endocytosis of antigen. Their dendritic morphology also allows them to make close contacts with many T cells at the same time. Immature DCs are phagocytic but are relatively inefficient at processing antigen. Stimulation with TLRs or cytokines leads to DC maturation resulting in the up regulation of class II MHC and co-stimulatory molecules. They also acquire the ability to migrate to lymphoid organs and remodel their endosomal compartments for antigen processing. Productive T cell activation requires a signal via the TCR-peptide-MHC interaction and also a second co-stimulatory signal. The best-characterized second interaction is CD28 which binds to its ligands, CD80 and CD86 on the APC (39). TCR binding to peptide-MHC in the absence of the second co-stimulatory signal leads to apoptosis or anergy of the T cell (40).

1.2.3 Processing of class I MHC binding peptides

Processing of peptides for binding to class I MHC is performed within the cytoplasm by the proteasome-ubiquitin system (41) (42). This is the main process by which cytoplasmic or nuclear proteins are degraded within a cell. Proteins are initially conjugated with ubiquitin, which then targets them to the proteasome for degradation (43) (44) (45). In addition to protein degradation, ubiquitinylation of proteins can control a wide range of cellular functions including protein activation, intracellular trafficking of proteins, regulation of signaling pathways and of DNA repair mechanisms (46) (47).

The oligopeptides produced by the proteasome are broken down into amino acids where they can be reutilized for protein synthesis (41). However, some short peptides escape further breakdown and are transported into the lumen of the ER via the specialized peptide transporter, transporter associated with antigen processing (TAP) (48). Within the ER, aminopeptidases trim peptides to 8-9 residues in length to produce peptides that can bind to class I MHC (49) (50) (51).

Prior to peptide binding, the class I molecules are stabilized by chaperone proteins within the ER such as Tapasin, calreticulin and ERp57 forming a peptide loading complex (52) (53). Tapasin also interacts with TAP and therefore helps to couple the translocation of peptides into the ER via TAP to peptide loading onto class I MHC (54). Tapasin also acts as a peptide editor by promoting the binding of high affinity peptides to class I MHC (55).

Following peptide binding, the chaperone proteins are released and the fully assembled peptide-MHC-I complex is exported to the cell surface (56) (Figure 1).

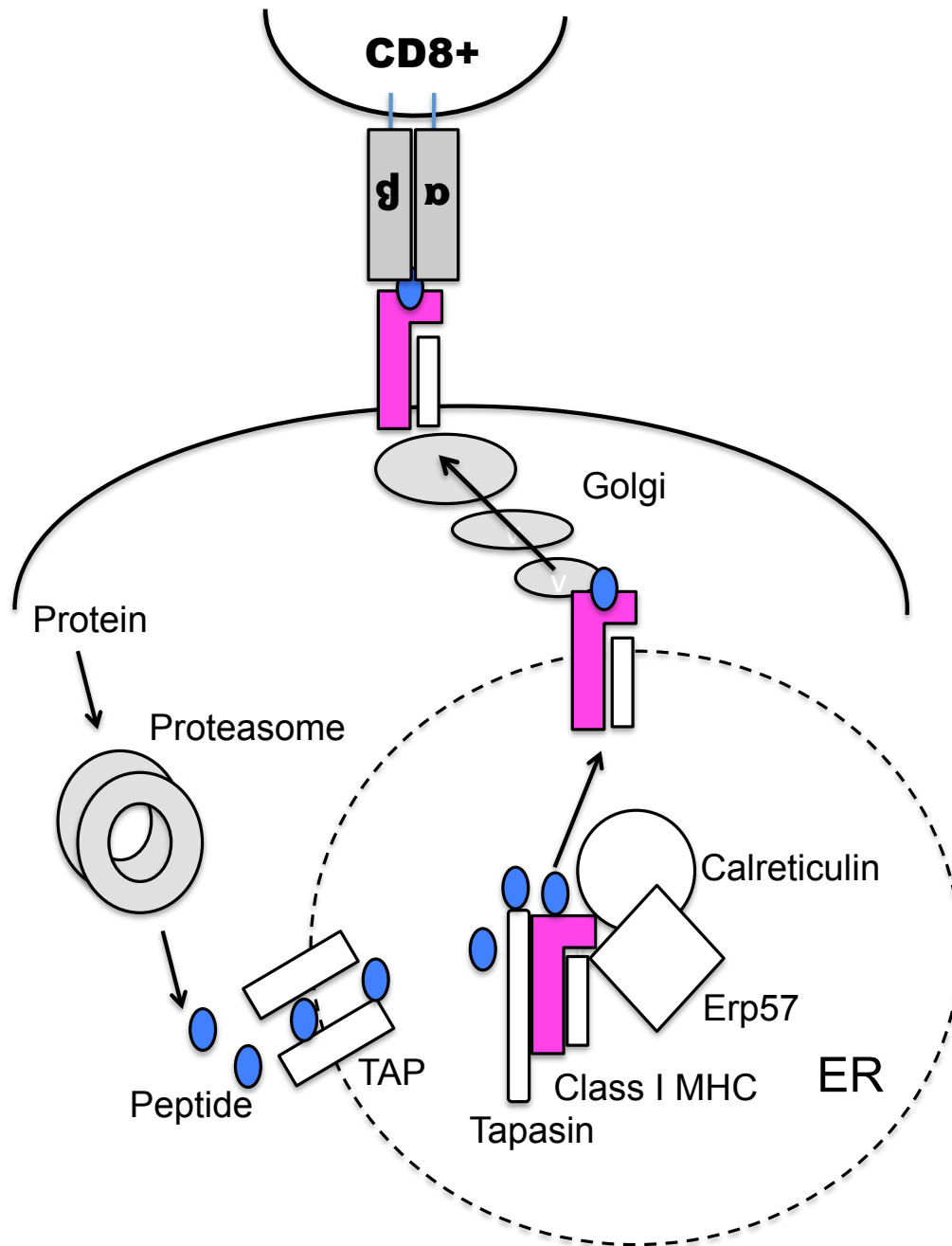


Figure 1 – Class I MHC antigen processing and presentation - Class I MHC molecules present peptides derived from endogenous cytoplasmic or nuclear antigens. Proteins are initially conjugated with ubiquitin and then targeted for processing by the proteasome. Short peptides generated by the proteasome are translocated into the ER by TAP where they are then loaded onto class I MHC. Prior to peptide loading, class I MHC molecules are stabilized by chaperone proteins such as Erp57, Calreticulin and Tapasin. Tapasin also binds to TAP and thus couples the translocation of peptides by TAP to loading onto class I MHC. Tapasin also acts as a peptide editor. Following peptide binding, the chaperone proteins are released and peptide-MHC class I are exported to the cell surface.

1.2.4 Cross Presentation

Cross Presentation is the process where exogenous antigens are taken up by professional APCs and presented by class I MHC. It is an important pathway for the presentation of tumour antigens by professional APCs. It was first described by Bevan et al in 1976 who demonstrated that minor histocompatibility antigens from transplanted allogeneic cells (e.g. exogenous antigens) could prime naïve CD8 T cells in a class I restricted manner (57). The presentation of exogenous antigen leading to CD8⁺ T cell activation is referred to as 'cross priming' whereas 'cross tolerance' refers to the induction of CD8 T cell deletion or anergy via presentation of exogenous antigen.

One of the major pathways of cross presentation is the cytosolic pathway, which is dependent on both the proteasome and TAP (58) (59). Exogenous antigens are taken up by phagocytosis and then transported from the phagosome into the cytoplasm for processing by the proteasome. There are a number of potential routes by which the proteins within the phagosome reach the cytoplasm: direct translocation into the cytosol; fusion of the ER and phagosome resulting in the protein being retro-trans located out of the ER by TAP. Once within the cytosol, exogenous antigen is processed by the proteasome and then follows the same route as endogenous antigens to be loaded onto class I molecules within the ER. The phagosome itself may act as a self-contained processing area containing TAP, class I MHC, Tapasin and other chaperone molecules. The fusion of the ER with the phagosome

allows donation of several essential processing structures to the phagosome (60) (61).

A pathway for cross presentation also exists that is independent of both the proteasome and TAP and is referred to as the vacuolar pathway (62). In the vacuolar pathway, exogenous antigen is degraded by cathepsin S following fusion of phagosomes with endolysosomes in a process called phagosome maturation (63) (64). Class I MHC molecules are loaded with peptide within the phagosome after trafficking into the phagosome either by internalization from the plasma membrane or by fusion of the ER with the phagosome. Both cytosolic and vacuolar cross presentation may occur within the same APC (62).

1.2.5 Processing of class II MHC binding peptides.

Peptides that bind to class II MHC are predominantly exogenous antigens acquired by professional APCs and degraded by the endosomal pathway. The α and β chains of class II MHC form heterodimers within the ER and then associate with the invariant chain (Ii). Ii acts as a chaperone to stabilize the heterodimer and also acts to accelerate the egress of class II molecules from the ER (65). Ii contains 2 di-leucine sorting motifs that direct the complex of MHC class II-Ii to a late endosomal compartment, called the MHC class II compartment (MIIC) (66). Within the MIIC, the Ii is digested by proteases until the residual class II associated peptide, CLIP, remains within the peptide-binding groove. CLIP acts as a surrogate peptide that prevents the premature

binding of peptides to class II MHC whilst it remains within the ER. Within the MIIC, a non-polymorphic class II related molecule HLA-DM (H2-^{DM} in mice) acts to facilitate and accelerate the exchange of the CLIP fragments for specific peptide. Following peptide loading, the MHC class II- peptide complex is transported to the plasma membrane to present peptide (Figure 2).

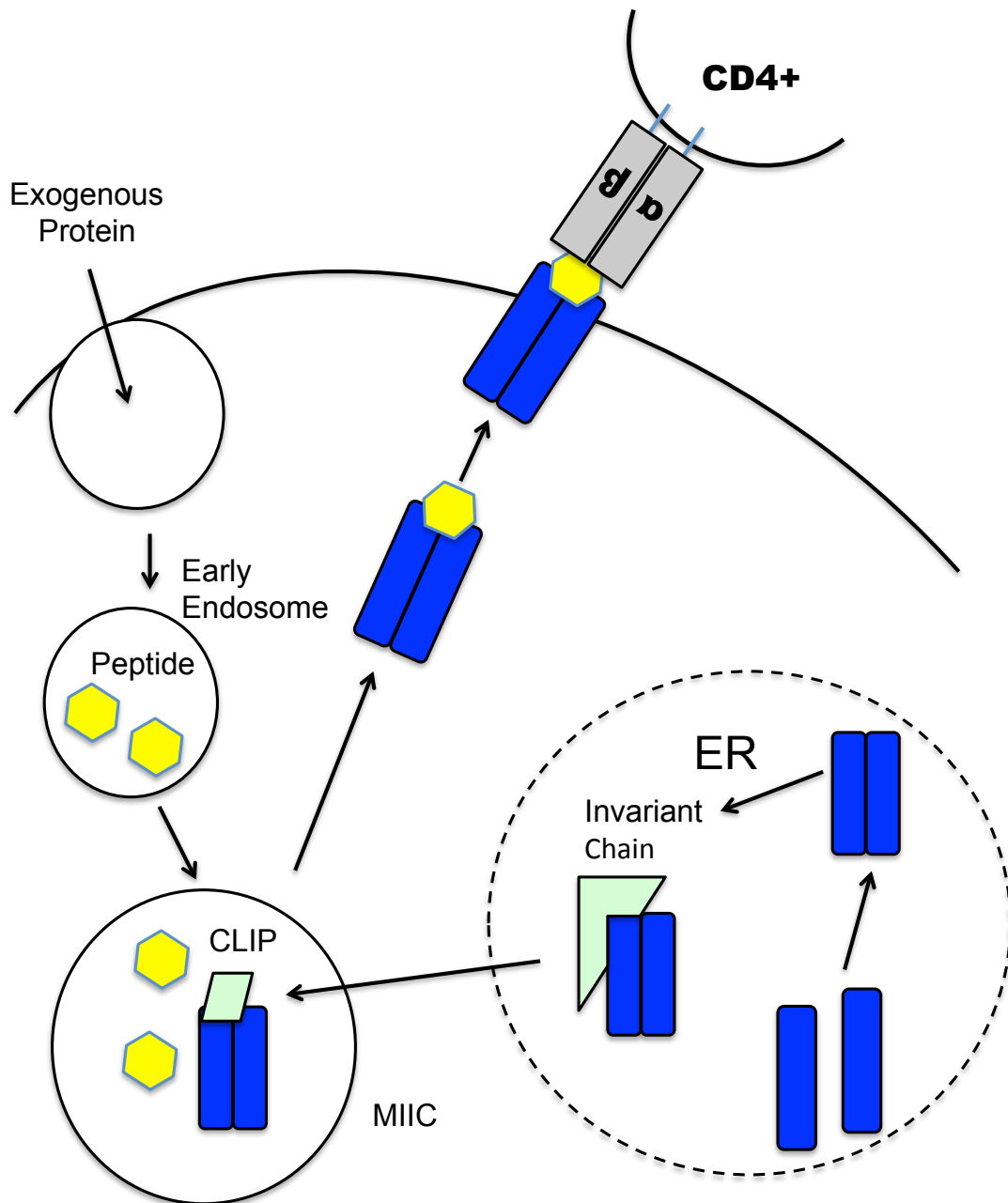


Figure 2 – Class II MHC antigen processing and presentation – Class II MHC binds peptides derived from exogenous antigens that are processed by the endosomal pathway.. The α and β chains of class II MHC form a heterodimer within the ER and then associate with the invariant chain (Ii) which acts as a chaperone protein to stabilize class II MHC. Ii contains 2 dileucine sorting motifs which targets the class II MHC to the late endosomal compartment, MIIC. Within the MIIC, the Ii is digested by proteases, leaving a residual class II associated protein, CLIP, within the peptide binding groove. Within the MIIC, HLA-DM acts to facilitate the exchange of CLIP for specific peptide. Following loading with peptide, class II-MHC-peptide is exported to the cell surface.

1.2.6 T cell trafficking

Prior to encounter of cognate antigen, naïve T cells migrate actively and in what appears to be a random fashion (67). Naïve T cells recirculate continuously between the lymphoid organs and the blood, increasing the probability of encountering cognate antigen. Binding to cognate antigen leads to a prolonged and functional interaction between the T cell and the APC resulting in arrest of T cell migration within the lymph node and proliferation of antigen specific T cells (68). Recently activated T cells exit the lymph node and then rapidly localize to areas of inflammation in order to combat pathogens or tumours. Targeted migration or homing of T cells is regulated by their differentiation state with the activation of T cells leading to altered expression of adhesion molecules and chemokine receptors. Importantly T cells will express different selectins and selectin ligands, chemokine receptors and integrins depending on the activation state of the cell.

Naïve T cells tend to migrate in a linear fashion from blood to lymph node to lymphatics and back into the bloodstream via the thoracic duct. The process by which T cells exit the blood stream and enter the lymph node via the high endothelial venules is a highly coordinated process. CD62L (L-selectin) is expressed by all naïve CD8⁺ T cells. It is the interaction between CD62L and its ligand that initiates the transmigration process of T cells out of the blood stream into the lymph node. To overcome the shear forces of the blood, naïve T cells must tether to the endothelium resulting in rolling of the T cells

along the high endothelial venules (HEV). HEV are a specialized post capillary vascular endothelium within lymph node paracortical regions. This rolling and tethering effect is mediated by the high avidity low affinity short lived interaction between CD62L on T cells and peripheral node addressins (PNAd) which are highly expressed in HEV (69) (70).

Following the tethering of the T cells, CCR7 on the T cell can be stimulated by the homeostatic chemokines CCL21 and CCL19 which are immobilized on the surface of the HEV (71). Signalling via CCR7 leads to a conformational change in and activation of LFA-1 (72) (73). Activated LFA-1 binds ICAM-1 leading to T cell arrest within the HEV and transmigration of the T cell into the T cell area of the lymph node, the paracortex.

Migration of T cells through the lymph node is controlled by sphingosine-1-phosphate (S1P) which binds S1PR1 expressed by T cells (74). S1PR1 is a G protein coupled receptor that activates signaling pathways within the T cells which control cell polarity and migration (75) (76). T cells are directed across a naturally occurring gradient of increasing S1P concentration which directs the exit of T cells from the lymph node to the efferent lymphatics (77) (78). If T cells migrate through a reactive node, the pro-inflammatory cytokine environment leads to down regulation of S1PR1 by the T cell leading to an increase in dwell time of activated T cells within the lymph node (79). Type I interferons induce expression of CD69 which inhibits the surface expression of S1PR1 (80). If T cells encounter their cognate antigen, TCR stimulation results in reduced S1PR1 transcription leading to retention of antigen specific

T cells within the lymph node (81) (82). Following activation or when inflammation is reduced, the expression of S1PR1 is regained through renewed transcription and via downregulation of CD69 (83) allowing exit of T cells from the lymph node.

Expression of chemokines, CCL21 and CCL19 within the T cell zone enhances T cell motility, facilitating scanning of APCs for cognate antigen. (84). Release of inflammatory cytokines and chemokines alters the lymph node architecture and thus modification of the homing ability of T cells. This also results in recruitment of additional T cell subsets and innate immune cells to the lymph node. Inflammation also increases microvascular blood flow to the lymph node thus increases the rate of T cell entry into the lymph node (85). Within the lymph node, there is competition for binding to antigen and as a result low affinity T cells exit the lymph node at earlier time points. Higher affinity T cells remain in contact with cognate antigen for longer and thus can clonally expand.

The trafficking patterns of T cells change with their transition from naïve to effector or memory T cells secondary to a shift in surface expression of proteins that regulate cellular trafficking. Naïve T cells constitutively traffic through lymphoid tissue whilst effector or memory T cells acquire the ability to enter non lymphoid sites where cognate antigen is located. Following activation, effector T cells down regulate CD62L and CCR7 which prevents these cells from gaining access to the lymph node via the HEV. In addition they upregulate the expression of homing molecules that will direct them to

non lymphoid tissues. Unique selectin and chemokine and integrin expression by the T cell will influence tissue specific homing of T cells following initial activation within the lymph node. Each tissue presents an organ specific molecular signature allowing entry of specific T cells expressing the relevant ligand (86). Different homing molecules are required for homing of T cells to the gut, skin or secondary lymphoid organs. Location of T cell priming may influence the homing characteristics e.g. T cells activated within lymph nodes draining the skin upregulate E-selectin and P-selectin ligands and CCR4 and CCR10 (87) whilst T cells that are found in the gut express $\alpha 4\beta 7$ and CCR9 (88). The multistep homing paradigm provides a model whereby unique combinations of selectin, chemokine receptors and integrins provide an organ specific area code and each tissue displays a specific and unique molecular signature which the T cell must recognize in order to gain entry to that tissue (86). This “imprinting” of T cells significantly influences their trafficking pattern but is not always a permanent state. T cells that have been imprinted within the gut or the skin can be reprogrammed following interaction with an APC from an alternative anatomical location. As memory T cells develop, the expression of CD62L and CCR7 can be upregulated. Effector memory T cells maintain a low expression level of CD62L and CCR7 whilst central memory T cells have high expression levels. Both effector memory and central memory T cells are found within the blood, spleen and peripheral tissues, central memory T cells are enriched within lymph nodes thus enabling memory T cells to maintain effective immunity within both the periphery and lymphoid organs.

1.3 THE TCR-CD3 COMPLEX AND CO-RECEPTOR FUNCTION

1.3.1 TCR structure

The $\alpha\beta$ TCR is a heterodimer composed of disulfide linked α and β chains, which share features with those of the Fab fragment of an antibody molecule. Both the TCR α and β chains are composed of a variable and a constant immunoglobulin like domain. The variable region is encoded by the rearranged V, D and J gene segments and the constant region is encoded by the constant gene segment (89). The complementarity-determining regions (CDR) of the variable domains are the area of the TCR that contacts peptide-MHC. The hypervariable CDR3 α and CDR3 β bind over the centre of the bound peptide whilst the germ line encoded CDR1 and 2 regions bind to the MHC molecule. CDR1 α and CDR1 β also make some contribution to peptide recognition by binding over the amino terminal and carboxy terminal peptide sequences respectively. Most $\alpha\beta$ TCRs bind diagonally across the peptide-MHC complex. TCRV β is positioned over the α 1 helix of the MHC and the TCRV α over the other MHC helix (α 2 in MHC class I or β 1 in MHC class II) (90) (91).

1.3.2 The immune synapse

T lymphocytes traffic constantly through the body, but following recognition of cognate antigen, their migration is arrested and a stable contact is formed between the T cell and APC. One of the initial steps following antigen

recognition is antigen induced upregulation of LFA-1 on the T cell which binds to ICAM-1 on the APC. This acts to slow T cell migration and initiate formation of the immune synapse. The immune synapse is a specialized structure that forms during the recognition of pMHC by TCR. It is composed of a bullseye structure containing a central aggregation of TCR-pMHC complexes termed the central supramolecular activation cluster (cSMAC) surrounded by a ring of adhesion molecules such as LFA-1, the peripheral supramolecular activation cluster (pSMAC) (92) (93). This structure forms within minutes of the initial TCR-pMHC contact and allows a stable synapse to form between the TCR and APC. TCR-pMHC microclusters form initially in the periphery and then translocate centrally into the cSMAC via an actin cytoskeletal dependent process. The peripheral TCR microclusters are the site of initiation of active T cell signaling and the formation of the cSMAC is not essential to initiate signaling (94) (95). The main function of the cSMAC may be to terminate signaling (96), facilitating the ubiquitination and internalization and degradation of the TCR (97). In addition to playing an important role in T cell priming, the immune synapse also provides a sealed point of contact to allow targeted delivery of cytolytic granules by effector T cells (98).

1.3.3 T cell co-receptor structure and function

Mature T cells that recognize antigen presented by class I MHC express CD8 whilst those that are class II restricted express CD4. Both CD8 and CD4 act as universal co-receptors, which can potentially bind to any polymorphic class I or II molecule respectively. CD8 and CD4 are structurally quite distinct.

CD4 is a single polypeptide composed of 4 extracellular immunoglobulin like domains (D1-D4). The crystal structures of the D1 and D2 domains of human CD4 and murine peptide-MHC II (I-A^k) demonstrate that the N terminus of D1 (of CD4) binds to the 2 membrane proximal domains of MHC class II ($\alpha 2$ and $\beta 2$) (99). The ternary crystal structure of TCR, peptide-MHC and CD4 (100) suggests that the TCR-MHC-CD4 complex forms an inverted V shaped structure where both TCR and CD4 are tilted inwards towards the MHC molecule, rather than pointing up vertically from the cell membrane. The MHC molecule is at the apex where the TCR and CD4 both bind MHC although TCR and CD4 do not come into direct contact. This suggests that there is a wide separation (70Å) between the membrane proximal TCR and the D4 domain of CD4 which may be the place where the CD3 complex sits. The movement of TCR or CD4 following MHC binding may produce a conformational change in CD3.

CD8 exists either as a CD8 $\alpha\alpha$ homodimer or a CD8 $\alpha\beta$ heterodimer. CD8 β requires association with CD8 α in order to be stably expressed on the T cell surface. Both CD8 α and CD8 β chains are membrane-anchored glycoproteins, which have an immunoglobulin like variable domain and a long stalk. CD8 $\alpha\beta$ binds to the $\alpha 2$ and $\alpha 3$ areas of the class I MHC, to an area distant from the TCR-peptide MHC interaction (101) (102). CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ are not interchangeable and both may have distinct functions. Coordinate binding of CD8 $\alpha\beta$ and TCR to MHC results in a much stronger signal than that produced by CD8 $\alpha\alpha$ (103) (104). In addition, CD8 $\alpha\alpha$ does not support the positive selection of class I restricted thymocytes during thymic

selection (105). In fact there is increasing evidence that CD8 α delivers an inhibitory signal. CD8 α is often expressed in conjunction with high affinity TCRs (106) (107) and the co-expression of CD8 α has been shown to decrease the functional avidity to TCR leading to reduced activation suggesting that it may act as a co-repressor (108) (109).

The exact role that CD4 and CD8 $\alpha\beta$ play in T cell activation is still being resolved but they are known to enhance TCR sensitivity for peptide. Binding of CD4 to class II MHC has been shown to increase cytokine production by CD4 T cells (110) and led to a marked reduction in the number of peptide-MHC required for T cell triggering (111). Preventing the binding of CD8 by mutating the $\alpha 3$ domain of MHC class I led to a reduction in specific tetramer binding and reduced T cell activation and reduced killing of target cells (112) (113). Garcia et al showed that the affinity of the TCR for the peptide-MHC was enhanced by CD8 due to a reduced dissociation off rate in the presence of CD8 (114). It has been suggested that CD8 acts to stabilize the interaction between peptide-MHC by increasing the half-life of binding or that CD8 binding to MHC promotes a more favorable docking conformation for TCR binding to peptide-MHC, or both. CD8 α associates with the src kinase, Lck via a conserved binding motif in the cytoplasmic domain of CD8 α (115) and acts to bring Lck into the vicinity of the TCR/CD3 complex. In addition, CD8 β has been shown to promote lipid raft association (116). Lipid rafts are ordered micro domains that are rich in cholesterol and sphingolipids. These rafts are stabilized by the presence of short saturated fatty acids such as palmitic or myristic acid. The cytoplasmic tail of CD8 β is palmitoylated which

allows it to efficiently partition into lipid rafts where it can associate with signaling molecules such as Lck and LAT which are also enriched in lipid rafts (117) (104). CD8 β enrichment in lipid rafts may enhance the active recruitment of TCR/CD3 complexes to the lipid rafts. Furthermore, deletion of the cytoplasmic tail of CD8 β reduced the association of CD8 with TCR/CD3 and led to a marked reduction of TCR binding to peptide-MHC (116) (118).

CD4 does not act to stabilize the interaction of TCR with MHC: blockade of CD4 binding has no effect on TCR-MHC affinity nor does it destabilize TCR-peptide-MHC binding (119). CD4, like CD8 α , associates with Lck via its cytoplasmic tail. CD4 is palmitoylated and is enriched in lipid rafts (120) and it is via these two actions that CD4 is thought to mediate co-receptor function.

CD8 or CD4 co-receptor binding to MHC is not an absolute requirement for T cell activation. High affinity TCR-peptide MHC interactions, such as in an alloreactive response, can be CD8 independent whilst weaker affinity interactions may be more dependent on co-receptor function to trigger a functional T cell response (121) (122).

The interaction between CD8 and MHC-I is of much lower affinity than that between TCR and peptide-MHC-I. The absolute requirement for CD8 binding to MHC-I may vary depending on TCR-pMHC-I affinity. Experiments using mutated pMHCI tetramers with altered CD8 binding properties demonstrated that binding of CD8 can profoundly affect TCR-pMHCI avidity. Class I MHC molecules with compromised CD8 binding were used to demonstrate that T

cell activation could not occur in the presence of weaker agonist antigens without CD8 co-receptor activation whilst T cell activation by strong agonists is only partially impaired by the loss of CD8 engagement (123). Using combinatorial peptide libraries and APCs that expressed HLA-A201 molecules which had differing CD8 binding affinities it was demonstrated that there was a direct positive association between the affinity of CD8 binding to MHC-I and the number of ligands it took to induce T cell activation (124). Increasing the affinity of the interaction between CD8 and MHC-I can lead to non specific T cell activation even in the absence of a functional TCR-pMHC interaction. Wooldridge et al generated a chimeric A2/K^b MHC molecule that increased the affinity of the pMHCI-CD8 interaction by >10 fold. This led to loss of tetramer binding specificity with tetramers binding to the T cell surface even in the absence of TCR expression. This non specific binding could be prevented by pretreatment with an anti-CD8 antibody which suggested that the loss of specificity for binding to pMHC-tetramer was mediated by CD8 and was occurring in a TCR independent manner. It appeared that the chimeric A2/K^b molecule was crosslinking the CD8 molecules leading to activation of the T cell in an antibody like manner (125).

Thus in situations of low antigen potency, CD8 plays a much greater role in increasing T cell antigen sensitivity whilst for stronger agonists, the contribution made by CD8 is much less. By extension of this theory it would be predicted that the CD8 co-receptor may act to increase the crossreactivity of T cells by increasing the range of agonist ligands to which T cells make functional responses. Binding of CD8 to MHC-I may provide a mechanism

whereby T cells can maintain their antigen specificity whilst achieving the desired level of sensitivity by the additional binding of CD8. CD8 may thus be essential to regulate the balance between an optimal level of cross reactivity and a broad antigen specificity of CD8⁺ T cells. CD8 function and the need for CD8 co-receptor interaction in both thymic education and in peripheral T cell activation may be dynamically increased or decreased *in vivo*. Mechanisms include the transcriptional inhibition of CD8 expression in double positive thymocytes (126), alternative expression of CD8 α isoforms, alterations in glycosylation pattern and selective internalization of CD8 following T cell activation (126) (127) (128) (129).

1.3.4 The TCR-CD3 Complex

CD3 forms a multimeric complex with the TCR and is responsible for the transmission of downstream signals from the TCR. It is essential for the stable expression of TCR on the cell surface. CD3 molecules are not expressed on the cell surface in the absence of TCR and have no ligand binding function in the absence of TCR (130). Signals transmitted via the TCR/CD3 complex are the primary checkpoint controlling T cell activation and the quality of the intracellular signal delivered, which determines the ultimate function and fate of the cell. The CD3 complex comprises 4 different chains: zeta(ζ), epsilon(ϵ), delta(δ) and gamma(γ). All chains contain a immunoreceptor tyrosine based activation motif (ITAM) (YxxL/Ix6–12YxxL/I) within their cytoplasmic domains (131) – each ζ chain contains 3 ITAMS, whilst the other three chains have one ITAM motif per chain.

CD3-TCR assembly takes place within the endoplasmic reticulum (ER) and is an ordered and well-controlled process. Incomplete or incorrectly assembled complexes are unable to leave the ER. The determinants for the correct assembly of the TCR-CD3 complex are contained primarily within the transmembrane and juxtamembrane domains of the individual chains. One of the initial steps is the pairing of CD3 ϵ with CD3 γ or CD3 δ resulting in formation of CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ heterodimers whilst CD3 ζ forms a CD3 $\zeta\zeta$ homodimer. CD3 $\gamma\epsilon$ preferentially associates with the TCR α chain and CD3 $\delta\epsilon$ preferentially pairs with the TCR β chain. The association between TCR and CD3 dimers is driven by charged residues within the transmembrane regions of the TCR and CD3 chains. Each of the CD3 chains has a single acidic residue within their transmembrane domain, which binds to a basic residue within the transmembrane region of the TCR α and TCR β chains (132) (133). The formation of a complete eight chain complex is dependent on the correct placement of these ionizable residues. Alanine substitution of any of these residues prevents the formation of a complete and correctly assembled TCR-CD3 complex. Conversely, correct assembly can still take place even if the transmembrane domains of the TCR are substituted entirely by polyleucine sequences as long as the basic residues remain in the correct position (134). Extracellular and intracellular domains are also not required for correct assembly as just the transmembrane and juxtamembrane sequences are sufficient to drive complex assembly. These basic and acidic residues contain signals for degradation should the individual subunits remain unassembled (135) (132). All of the CD3 chains contain one or more ER

retention motifs. It has been proposed that following the final assembly step of binding of the CD3 $\zeta\zeta$ homodimer, the ER retention motifs are masked. This then allows export of the fully assembled TCR-CD3 complex to the cell surface (136) (137) (138). If one or more components are absent then the incomplete complex is retained within the ER and degraded. The final composition of the CD3 complex is one TCR $\alpha\beta$, one CD3 $\gamma\epsilon$ heterodimer, one CD3 $\delta\epsilon$ heterodimer and one CD3 $\zeta\zeta$ homodimer (132) (Figure 3).

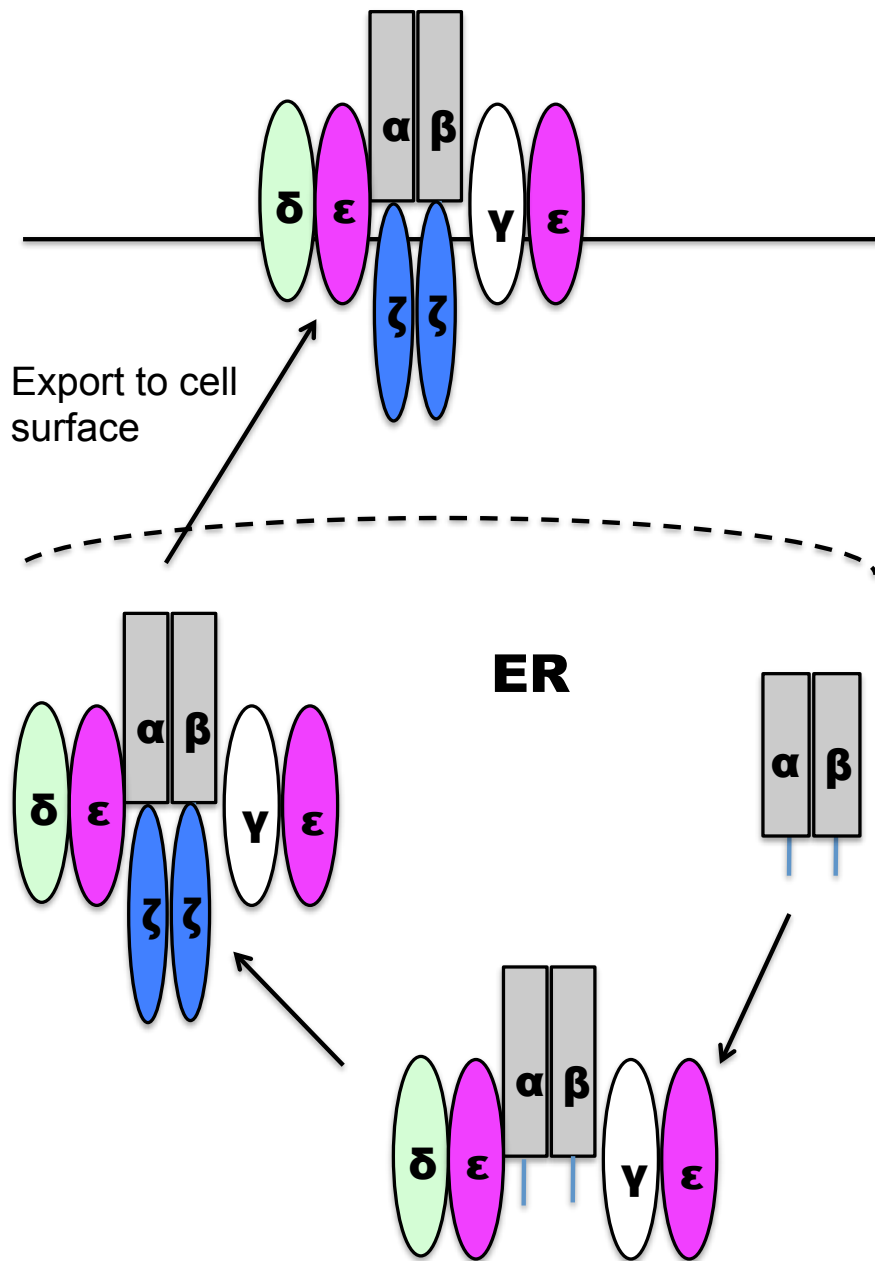


Figure 3 – Assembly of TCR and CD3 complex. The TCR-CD3 complex is a multichain complex and complete assembly with all chains is required prior to cell surface expression. TCR-CD3 assembly takes place within the ER and takes place in an ordered sequence. Following pairing of the TCR α and β chains to form a $\alpha\beta$ TCR heterodimer, CD3 ϵ then pairs with either CD3 γ or CD3 δ to form CD3 $\gamma\epsilon$ or CD3 $\delta\epsilon$ heterodimers. CD3 $\delta\epsilon$ preferentially pairs with the TCR α chain whilst CD3 $\gamma\epsilon$ preferentially pairs with the TCR β chain. CD3 ζ forms a CD3 $\zeta\zeta$ homodimer and one of the final steps is binding of the CD3 $\zeta\zeta$ homodimer prior to export to the cell surface. Any incorrectly assembled complexes are retained in the ER and degraded.

Initial experiments to study signaling events initiated by the TCR-CD3 complex were completed using chimeric molecules where the cytoplasmic domains of individual CD3 chains were fused to extracellular and transmembrane domains from other cell surface proteins (139) (140) (141). These chimeric proteins were then expressed in T cells that had been selected on the basis of absence of TCR expression. Antibody crosslinking of the extracellular domain of the chimeric molecule triggered all downstream TCR mediated signaling events leading to T cell activation. However, mutation of the tyrosine residues within the ITAM motifs prevented activation of the cell following antibody crosslinking. These findings demonstrated that tyrosine phosphorylation of the CD3 ITAMS was an essential early step for T cell activation. This early ITAM phosphorylation is performed by src protein tyrosine kinases (PTK), Lck and Fyn. These phosphorylated tyrosine residues within the ITAMs act as docking sites for the recruitment of signaling proteins to the TCR complex forming a scaffold for downstream signaling. The two phosphorylated ITAMS within CD3 $\zeta\zeta$ act as a binding site for the tandem SH2 domains of ZAP-70 (142). ZAP-70 undergoes a conformational change following binding to CD3 $\zeta\zeta$, which leads to its activation (143). Activated ZAP-70 then recruits and activates linker for the activation of T cells (LAT) and SLP-76. LAT and SLP-76 recruit and activate a key signaling molecule, Phospholipase C- γ (PLC- γ). PLC- γ triggers three further downstream signaling pathways leading to activation of transcription factors, NF κ B, NFAT and AP-1 triggering T cell activation and proliferation.

It is likely that the entire complement of ITAMS contained within the CD3 complex is required for normal T cell development and effector function. Holst et al generated mice that expressed differing ratios of wild type and mutant ITAMS within the CD3 complex. They showed that mice with less than 7 functional ITAMS within the TCR-CD3 complex developed lethal autoimmunity. This was thought to be secondary to a lower signal strength, which led to a failure to delete self reactive T cells in the thymus. There was a linear correlation between the level of T cell proliferation and the number of wild type ITAMS although cytokine responses were not affected by the number of ITAMS present (144). This gave rise to the concept of “scalable signaling” where a range of possible responses can be generated following TCR engagement. The main function of having multiple ITAMS thus appears to be to control the strength of the TCR signal. Differing ITAMs within the individual chains may serve distinct functions and individual ITAMS may be responsible for recruitment of discrete and different signaling molecules and this may influence downstream signaling pathway recruitment and the quality of the signal (145).

1.3.5 Models of TCR signaling

It is still incompletely understood how the binding of TCR to peptide-MHC leads to the initial phosphorylation of ITAMs within CD3. Phosphorylation of ITAMS must be tightly controlled to prevent inappropriate activation of the T cell. It is thought that one control mechanism is the sequestration of the cytoplasmic tails of CD3 ϵ and CD3 ζ within the plasma membrane when the T

cell is in a resting state. The cytoplasmic domains of CD3 ϵ and CD3 ζ have a net positive charge due to stretches of basic amino acids and these interact with the negatively charged lipid bilayer of the plasma membrane. The key tyrosine residues are therefore buried within the interior of the lipid membrane and when in this position cannot be phosphorylated by Lck. TCR ligation results in release of the cytoplasmic tails exposing the ITAMs for phosphorylation (146) (147).

It is not clear what drives the release of the cytoplasmic tails from the membrane following TCR ligation. TCR aggregation has been proposed as a possible model of T cell activation. TCR aggregation would lead to very close apposition of CD3 ITAMS and protein tyrosine kinases leading to transphosphorylation. In addition, receptor multimerization may lead to local competition for binding to acidic residues within the lipid membrane and as a result some CD3 ϵ and CD3 ζ tails would be released from the membrane. Models based on conformational change of the TCR have also been proposed. A mechanical force may be transmitted across the membrane following binding of TCR to peptide-MHC leading to a conformational change of the TCR that is transmitted to the CD3 complex (148) (149) (150). The kinetic segregation model (151) proposes that upon contact with the APC, proteins are segregated within the lipid membrane. This is partially due to size differences and steric restraints of differing proteins. TCR triggering induces a signaling event, which is amplified and maintained due to exclusion of large bulkier protein tyrosine phosphatases such as CD45. The tyrosine phosphorylation is thus promoted and maintained due to phosphatase

exclusion. A similar model proposes that individual TCRs are retained in an inactive conformation within positively charged areas of the cell membrane due to constraint provided by the actin cytoskeleton (152). Coupling of TCR phosphorylation and signaling to the actin cytoskeleton redistributes the TCR to a more ordered area of the plasma membrane, which excludes larger molecules and phosphatases such as CD45. Most of these models of TCR triggering are not mutually exclusive and it may be that elements of all of them are important.

1.4 PRINCIPLES OF TCR GENE THERAPY

1.4.1 Overview of TCR gene therapy

The production of TCR transduced T cells initiates with the isolation a T cell clone that expresses a TCR that is specific for a defined target antigen/MHC complex. The T cell clone is normally obtained by culturing TILs or peripheral blood mononuclear cells (PBMCs) in the presence of APCs that have been pulsed with the target epitope presented by a defined HLA allele. High affinity T cell clones can be isolated using MHC-peptide tetramer staining and/or their ability to recognize and lyse target cells that have been pulsed with low concentrations of cognate peptide/MHC. Once a high affinity clone has been selected, the genes encoding the α and β chains of the TCR are cloned into a retroviral vector. These vectors are then transfected into packaging cell lines which produce TCR retroviral vector particles which are used to transduce polyclonally activated T cells. T cell activation is an essential step in the

transduction process as retroviral vector genes can only integrate into the genome of actively proliferating cells. TCR α and β chains are then generated by the transduced T cell and a novel TCR expressed on the cell surface, which can be detected by flow cytometry within 24 hours of transduction. These TCR transduced T cells can then be adoptively transferred to patients to target defined antigens (Figure 4).

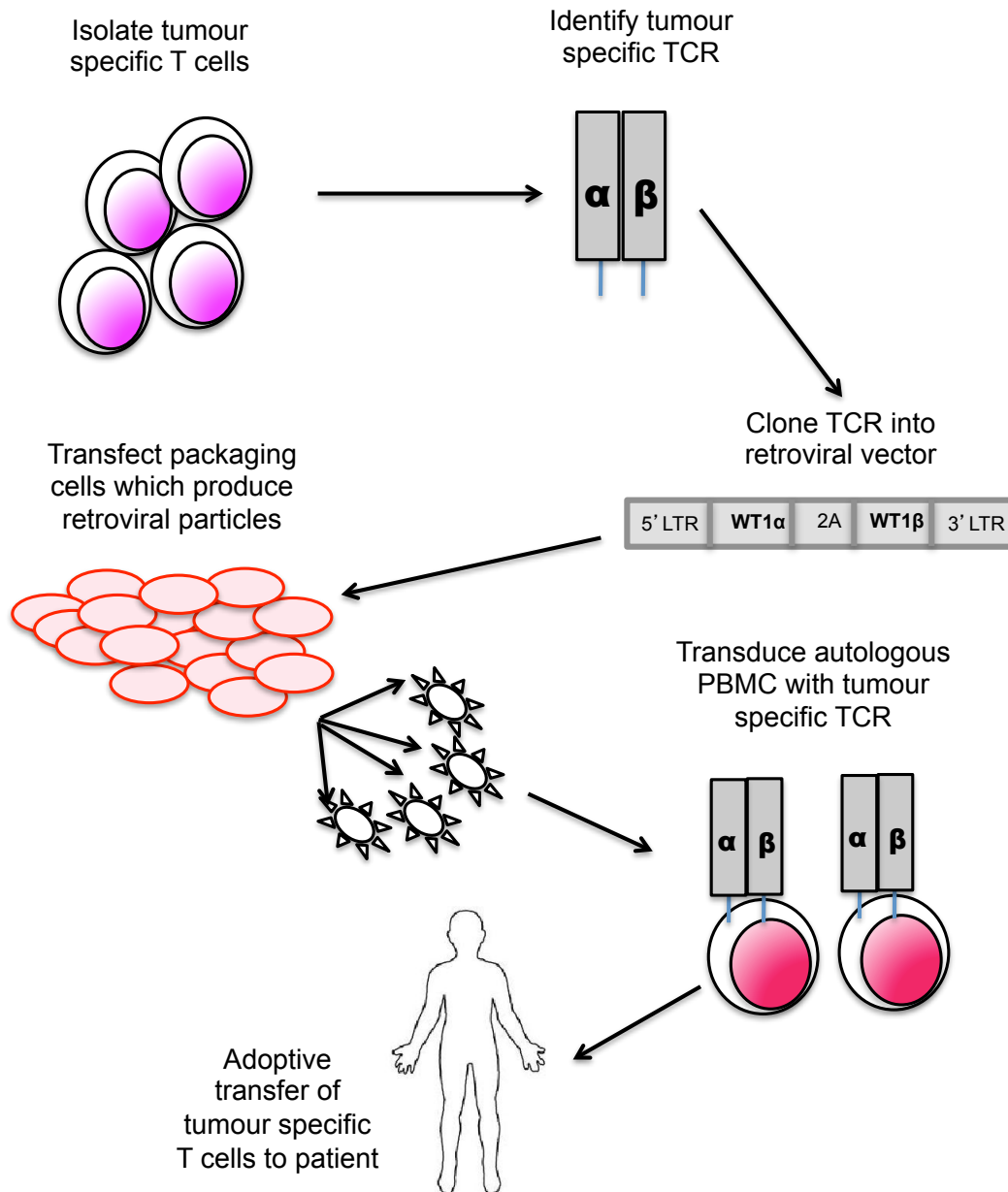


Figure 4 – Generation of tumour specific TCR transduced T cells for adoptive immunotherapy. The production of tumour specific TCR transduced T cells initiates with isolation of a T cell clone that expresses a TCR of defined specificity for peptide-MHC. These T cell clones can be obtained by culturing TILs or PBMCs with APCs of a known MHC haplotype that have been pulsed with a specific peptide. Once a high affinity clone has been selected the genes encoding the α and β chains of the TCR are isolated and then cloned into a retroviral vector. The retroviral vectors are then transfected into packaging cell lines which produce active retroviral particles that are then used to transduce a patient's autologous PBMCs. T cells expressing the introduced TCR can then be adoptively transferred to patients to target tumour epitopes.

1.4.2 Retroviral vectors

Retroviral vectors are excellent agents for gene delivery into somatic cells. During the retroviral life cycle, the retroviral genome is integrated into the host genome as a provirus. As a result, genes inserted into retroviral vectors will be stably expressed for the life span of the host cell even through cell divisions. In our research, the retroviral genome is replaced by genes encoding the α and β chains of the TCR. As a result, retroviral vectors lack the viral genes that are required for replication and thus are replication incompetent.

The elements required for the production of complete retroviral particles can be provided in *trans* within packaging cells. Packaging cell lines are stably transfected with defective helper viruses containing the retroviral genes *gag*, *pol* and *env* which encode viral core proteins, core enzymes and envelope glycoproteins however they do not contain the relevant packaging signal. The sequences required for integration, gene expression and the packaging of genomic RNA are provided in *cis* with the retroviral vector. The minimal structural features that are required in *cis* are both long terminal repeats (LTRs), the primer binding sites and the packaging signal (153) (154). The retroviral vector is transfected into the packaging cell line, which package the retroviral particles and secrete them into the supernatant, which is harvested and used to transduce host cells such as T lymphocytes. These replication defective virions can only carry out one more round of infection and

integration as they lack the viral genome and therefore can't go onto produce further active retroviral particles.

1.4.3 Strategies to improves TCR gene therapy

Whilst adoptive transfer of TILs in patients with metastatic melanoma has resulted in excellent clinical responses, for the majority of malignancies it is not possible to isolate tumour reactive lymphocytes in sufficient number. As the majority of tumour antigens are self antigens and thus widely expressed within self tissues, the majority of high avidity tumour specific T cells will have been deleted during thymic selection or will be regulated by peripheral tolerance mechanisms. As a result tumour reactive T cells can't be isolated or only very low avidity T cells can be found within the autologous repertoire. One of the advantages of TCR gene therapy is that it allows the generation of tumour specific T cells that are absent from the endogenous T cell repertoire.

One method to isolate high avidity tumour specific TCRs for TCR gene therapy utilizes transgenic mice that express human HLA molecules. These transgenic mice can be immunized with human tumour epitopes and the murine T cells that bind the tumour epitope with high avidity can be selected and the TCR genes isolated (155) (156). These TCRs thus will recognize human derived tumour peptides presented by human HLA molecules. Alternatively, the allorestricted approach can be used to generate high avidity T cells in vitro. Here, human PBMCs are stimulated with allogeneic APCs that have been pulsed with tumour peptides. From these, the tumour specific T

cells can be isolated from the purely allospecific responders (157). Using both of these methods, tumour specific TCRs that would ordinarily be absent from the normal peripheral T cell repertoire can be isolated and used for retroviral transduction.

Since the initial studies utilizing TCR gene modified T cells there have been a number of improvements all of which should lead to enhanced efficacy when translating this technique into clinical practice. These include improvements in vector design to enhance TCR surface expression, increasing the affinity of the TCR, selecting different T cell populations for transfer and utilizing conditioning therapy to enhance persistence of TCR transduced T cells following adoptive transfer.

1.4.4 Increasing TCR surface expression

The avidity of a TCR expressing T cell is dependent on the individual affinity of TCR for peptide-MHC and also how much TCR is expressed on the cell surface. When a new TCR is introduced into a T cell, both the introduced and endogenous TCR are expressed on the cell surface. One of the challenges of TCR gene therapy has been how to increase the surface expression of the introduced TCR. Increased surface expression of TCR correlates with increased sensitivity for the target peptide-MHC and increased anti tumour activity in vivo (158) (159) (160).

The amount of introduced TCR that is expressed on the cell surface is dependent on both the introduced TCR and the endogenous TCR. The transduction of a “strong” TCR results in a higher surface expression at the expense of a “weak” endogenous TCR. It is thought that TCR strength is related to the intrinsic pairing properties of the alpha and beta chains with one another and their ability to associate with CD3 within the cell (161). A “strong” transduced TCR will more effectively compete with and bind to the available CD3 than a “weak” endogenous TCR. Introducing a TCR of equal strength to the endogenous TCR will result in dual expression of the two TCRs on the cell surface.

One of the rate limiting steps for expression of the transduced TCR is competition with the endogenous TCR for binding to CD3. Ahmadi et al transduced TCR and additional CD3 molecules into murine CD8⁺ T cells. Co-transduction of TCR and CD3 led to enhanced surface expression of the introduced TCR and increased binding to specific pentamer. This led to increased functional avidity in vitro compared to CD8⁺ T cells transduced with TCR alone. Following adoptive transfer into tumour challenged irradiated mice, tumour specific TCR + CD3 CD8⁺ T cells provided superior tumour protection in vivo than TCR only CD8⁺ T cells. They underwent greater expansion in vivo and trafficked to the tumour bed faster and in higher numbers and also had superior recall responses (5).

When a novel TCR is introduced, there is potential that the introduced TCR will mispair with the endogenous TCR chains. The introduced TCR- α and - β

chains may form mixed heterodimers of unknown specificity with the transduced TCR- α chain forming a heterodimer with the endogenous TCR- β chain and the transduced TCR- β chain forming a heterodimer with the endogenous TCR- α chain. This can result in the expression of 4 different TCR α - β heterodimers by an individual transduced T cell. This results not only in a marked reduction of the introduced TCR on the cell surface but also has the potential to generate T cells which express auto reactive TCRs which have not undergone negative selection in the thymus (Figure 5).

There are now a number of strategies to prevent mispairing which act to increase the surface expression of the introduced TCR. TCR- α and - β chains are normally covalently linked by a single disulphide bond between their constant regions. TCR- α and - β chains have been genetically engineered to express a second disulphide bond within the constant region, which enhances the preferential pairing of the introduced TCR- α and - β chain with each other. This has been shown to reduce mispairing and increase the functional avidity of transduced T cells (162) (163) (164). Human TCR- α and - β chains have also been altered so that the human constant region is replaced by the murine constant region. This has also been shown to lead to enhance preferential pairing of the modified TCR- α and - β chains leading to increased surface expression of the TCR. In addition, the murine constant region binds more efficiently to human CD3 components and can thus compete for CD3 binding more efficiently than the endogenous TCR (164).

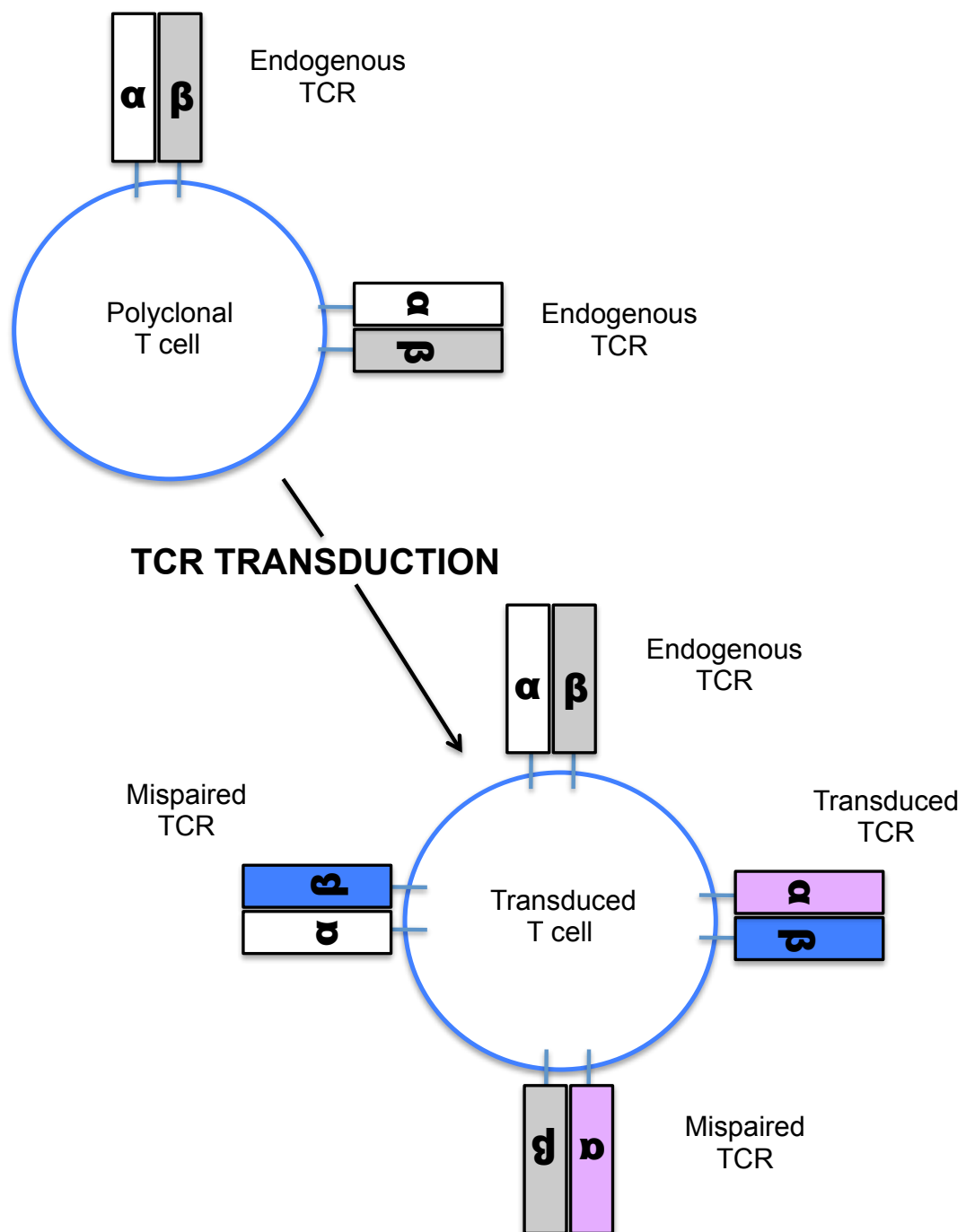


Figure 5 – TCR mispairing. When a novel TCR is introduced into a polyclonal T cell by TCR transduction it can potentially result in the expression of 2 novel TCR of unknown specificity on the T cell surface. The α chain of the introduced TCR can pair with the β chain of the endogenous TCR and the α chain of the endogenous TCR can pair with the β chain of the introduced TCR. This potentially can lead to generation of auto reactive T cells and also results in reduced surface expression of the introduced TCR. There are a number of strategies in use to reduce or prevent mispairing such as introduction of a novel disulphide bond, murinization of the TCR constant regions and using zinc finger nucleases to down regulate the expression of the endogenous TCR.

Careful in vitro experiments have demonstrated that combining the introduction of an additional disulphide bond and murinization of the TCR constant regions has an additive effect on reducing mispairing and increasing surface expression of the introduced TCR although neither completely eliminates mispairing (162).

An additional TCR modification explored the replacement of the TCR constant domains with the human CD3 ζ molecule. Similar to murinization of the constant region, such modified TCR chains are unable to mispair with the endogenous TCR chains and only pair with each other (165) (166). In addition, TCR $\alpha\beta$:CD3 ζ does not compete with the endogenous TCR $\alpha\beta$ for binding to endogenous CD3. The intracellular assembly and surface expression of the TCR: ζ fusion is completely independent of CD3 γ,δ or ϵ . When compared to transduction of unmodified TCR $\alpha\beta$ chains, the modified TCR: ζ showed higher surface expression and could transduce downstream signaling from the TCR in response to antigen specific cells. It was shown that the TCR: ζ mediated potent activation of NFAT but it was not clear if all the normal signaling functions of the TCR/CD3 complex were retained. The TCR: ζ construct lacks the extracellular connecting peptide motif, the transmembrane domain and the intracellular domain of the TCR chains, which may be involved in calcium mobilization, the NF- κ B pathway and control TCR down regulation (167) (168) (169). In addition the TCR: ζ construct lacks the ITAMs of the CD3 γ,δ and ϵ chains, which is likely to affect the T cell function. It was demonstrated that the TCR: ζ was able to form immunological synapses following antigen recognition that was independent of the endogenous TCR-

CD3 complexes. The TCR:ζ closely associated with CD8α and class I MHC and co-localized with CD28 and CD45 in lipid rafts in a similar fashion to wild type TCRs (170).

Expression of the introduced TCR may also be increased by suppression of endogenous TCR expression. This has been achieved by the use of zinc finger nucleases to target and disrupt the endogenous TCR-α and -β chain genes. Zinc finger nucleases contain zinc finger DNA binding domains and an endonuclease. They can be designed to target and disrupt specific DNA sequences. Following DNA binding, the endonuclease element introduces a DNA double strand break, which is then repaired by non homologous end joining leading to the random insertion and deletion of nucleotides and thus targeted gene disruption. This strategy has been used to prevent expression of the endogenous TCR-α and -β chain genes prior to introduction of a tumour specific TCR. Human T cells were initially transduced with vectors encoding zinc finger nucleases and these 'edited' T cells, lacking CD3 in the absence of endogenous TCR, were then selected and transduced with a vector encoding the WT1-TCR. WT1-TCR transduced edited T cells had high surface level expression of introduced WT1-TCR. This resulted in enhanced activity against relevant target cells and also reduced non specific alloreactivity due to prevention of mispairing. In a humanized mouse model, infusion of WT1-TCR transduced T cells that had normal endogenous TCR expression resulted in lethal GVHD in all mice as a result of mispairing of the introduced TCR and endogenous TCR chains. This was in contrast to the WT1-TCR edited T cells that did not produce GVHD in any mice following infusion. WT1-TCR edited T

cells also displayed tumour efficacy in vivo following challenge with a primary human leukaemia and could persist in vivo for at least 28 days post transfer (171).

Improvements in vector design have led to an increase in transduction efficiency and thus higher TCR surface expression. Codon optimization to replace rare codons within the TCR genes with those in more frequent use has been shown to increase the expression level of the optimized TCR compared to wild type. This, combined with removal of mRNA instability motifs and cryptic splice sites, leads to enhanced stability and translation of mRNA and thus higher levels of TCR transgene expression (160) (172). Use of Internal ribosomal entry sequences (IRES) or 2A sequences also allows the delivery of both the TCR- α and TCR- β chains within the one vector. The IRES is a distinct nucleotide sequence that allows translation initiation to occur in the middle of an mRNA sequence which results in the production of multiple proteins from a single mRNA transcript. The IRES is placed between the TCR- α and TCR- β chain with translation of the upstream alpha gene initiating at the 5'cap. Translation of the downstream TCR- β gene occurs in a cap independent manner following binding of the ribosome to the IRES (173). Use of viral derived 2A sequences may be even more effective than IRES sequences at delivering expression of equimolar amounts of the TCR- α and TCR- β chains (174) (175). The 2A sequence is placed between the TCR- α and TCR- β genes and allows multiple proteins to be generated from a large polyprotein that has been encoded by a single open reading frame. As the polypeptide is synthesized, the last amino acid of the 2A sequence prevents

peptide bond formation but without arresting translation. The upstream polypeptide is released with the 2A sequence fused to its C terminus but the ribosome remains on the mRNA and translates the downstream sequence. This leads to production of nearly stoichiometric amounts of both the TCR- α and TCR- β chains produced from a single open reading frame. Direct comparison of retroviral vectors with IRES or 2A sequences linking the TCR- α and TCR- β chains, has demonstrated a higher level of TCR gene expression when utilizing 2A sequences with resulting improved T cell function (176).

1.4.5 Increasing TCR affinity

As a result of thymic selection, circulating peripheral T cells express TCRs that have low affinity for their cognate peptide-MHC, usually within the range of 1-100 μ M (177) (178). The affinity of individual TCRs can be increased by making amino acid substitutions within the CDRs. Using bacteriophage or yeast display technology, high affinity TCRs can then be selected (179) (121) (180). This can lead to generation of tumour specific TCRs with affinity within the nanomolar or picomolar range. With very big increases in affinity, some TCRs do appear to lose antigen specificity and may potentially become autoreactive (121). TCR function may also start to decrease when affinity is increased above a certain level. Upon binding to peptide-MHC, high affinity TCRs have much longer dissociation times. This will lead to a reduction in the number of TCRs that can bind to that peptide-MHC which may decrease T cell activation. A comparison of high affinity and wild type TCR showed that whilst the high affinity TCR triggered much faster effector responses, there

was a loss of response in the presence of low density antigens as affinity increased (181). Rational design of affinity matured TCRs thus should focus on production of TCRs that only have modest improvements in affinity compared to the wild type TCR rather than aiming for ultra high affinity TCRs.

1.4.6 Enhancing persistence of adoptively transferred TCR transduced T cells

Using TCR transduced T cells to deliver effective anti tumour responses may be dependent on persistence of the transduced cells and the generation of recall responses. Studies using adoptive transfer of TILs in melanoma patients have shown that increased persistence of TILs correlated with enhanced anti melanoma responses (182). Multiple rounds of in vitro stimulation of tumour specific T cells typically leads to a population of T cell that predominantly have an end stage effector phenotype. This may adversely affect their ability to persist post transfer, leading to reduction in anti- tumour responses (183) (184). The transfer of naïve or memory T cell populations may be more efficacious at providing tumour protection and there is growing evidence to support this.

Central Memory CD8⁺ T cells maintain high levels of CD62L and CCR7 and can efficiently recirculate through secondary lymphoid organs where they encounter APCs. Effector memory CD8⁺ T cells have down regulated the expression of CD62L and thus preferentially recirculate in peripheral tissues. Effector memory T cells have lower levels of expansion on antigen

rechallenge compared to central memory T cells (184) (185). Klebanoff et al showed that adoptive transfer of tumour specific central memory T cells led to more effective anti-tumour responses than the transfer of effector memory T cells (184). Following adoptive transfer into unconditioned primates, CD8+ CMV-specific effector T cells derived from central memory T cells had enhanced persistence in vivo compared to effector T cells derived from effector memory precursors. These central memory derived effector T cells could migrate to secondary lymphoid organs and they had the ability to revert back to either a CD62L- or CD62L+ memory cell phenotype (186).

The most effective T cell population for adoptive immunotherapy may be naïve T cells. Using a pmel-1 TCR transgenic mouse model (where T cells were specific for a melanoma antigen, gp100, also expressed by self tissues) the adoptive transfer of CD62L+ CD44^{low} CD8+ naïve T cells into mice resulted in faster clearance of established melanoma compared to the transfer of central memory CD62L+ CD44^{high} CD8+ T cells. This superior tumour protection was associated with greater in vivo expansion and production of greater levels of IFN-γ and IL-2 post transfer (187).

Lymphodepletion of patients with chemotherapy and/or irradiation is very effective at promoting the expansion of the adoptively transferred T cell population. In patients with melanoma, the administration of conditioning therapy to induce lymphopaenia prior to adoptive transfer led to an increase in persistence of the transferred T cells (182) (188) (10). Similarly in mouse models, increased levels of lymphodepletion have been associated with

increased persistence of adoptively transferred T cells and superior tumour clearance (189). Lymphodepletion drives homeostatic proliferation of residual peripheral T cells that reconstitute the peripheral T cell pool (190) (191). The same lymphopaenia driven homeostatic proliferation also drives expansion of adoptively transferred T cells (192). The homeostatic cytokines, IL-7 and IL-15 and also interactions between TCR and self-peptide-MHC are thought to promote this proliferation (178) (193) (194) (195). The amount of proliferation of the adoptively transferred T cells is limited by the amount of IL-7 and IL-15 and the amount of self-peptide-MHC that is available. Cyto-reduction of the host haematopoietic system ensures that the adoptively transferred T cells do not have to compete for access to homeostatic cytokines, thus promoting their expansion. T cells with a higher affinity for self peptide-MHC may also have an additional proliferative advantage, which may benefit tumour specific T cells as many tumour epitopes are over expressed self peptides.

1.4.7 Potential limitations of TCR gene therapy

Mispairing of TCR α and β chains not only reduces the surface expression of the transduced TCR but may lead to potential harmful effects. Mispairing generates TCRs of unknown specificities that have not been subjected to negative selection in the thymus and are thus potentially autoreactive. Bendle et al have shown that the adoptive transfer of transduced T cells can lead to graft versus host disease post transfer. This was demonstrated to be

secondary to the formation of de novo TCR heterodimers targeting self tissues (196).

On target/off tumour effects of TCR transduced T cells can also produce harmful effects following adoptive transfer which has been demonstrated in a number of clinical trials. In patients with colonic cancer who received adoptive transfer of T cells transduced with a high affinity CEA-specific TCR, 3/3 patients treated developed a transient severe colitis. This was associated with a fall in levels of CEA which is overexpressed in many epithelial cancers but is also expressed at lower levels in normal tissues. 1/3 patients in this trial had a reduction in tumour burden (197). A clinical trial treating patients with metastatic malignancies with the adoptive transfer of T cells transduced with a high avidity anti-MAGE-A3 TCR led to severe and unexpected on target toxicities (198). MAGE-A3 is a cancer testes antigen, a group of tumour antigens expressed in a variety of common epithelial tumours including melanoma, breast and lung cancer but which have very restricted expression in germ line tissues such as the adult testes or in placenta and trophoblasts making them ideal targets for tumour immunotherapy. In this trial, 5/9 patients had cancer regression but unexpectedly 3/9 patients developed severe neurological toxicity within 1-2 days post infusion, 2 of whom subsequently died. MRI scans of their brain demonstrated necrotizing leucoencephalopathy with extensive white matter defects associated with brain infiltration by the transduced T cells. This was thought to have arisen due to recognition of the MAGE-A12 protein by the anti-MAGE-A3 TCR which is also known to bind to epitopes of MAGE-A9 and A12 in addition to MAGE-A3. MAGE-12

expression was detected in brain sections from both normal and patient samples although this had previously not been described in human brain samples. Targeting of MAGE-A12 by the TCR transduced T cells was thought to trigger the inflammatory response leading to the neurological toxicity. Patients who went on to develop neurological toxicity had received a higher total number of cells and higher number of CD3+/CD8+/Tetramer+ T cells and also a higher percentage of cells with a naïve phenotype. These studies highlight the need for careful selection of TCR, particularly when using high avidity engineered TCRs that are directed against antigens that are overexpressed in tumour but are also expressed at low levels in normal tissues.

Engineering T cells to express suicide genes in addition to TCR allows transduced T cells to be removed should toxicities arise post transfer. The suicide gene encodes a protein that acts to convert a non toxic prodrug into a toxic metabolite, thus the suicide gene can be switched on by the administration of the prodrug. The Herpes simplex thymidine kinase (HSV-TK) is the most extensively studied suicide switch and has been validated in clinical trials. HSV-TK phosphorylates the prodrug ganciclovir to form triphosphate ganciclovir which is incorporated into elongating DNA strands ultimately leading to the selective death of transduced cells. It has been used in patients post allogeneic haematopoietic stem cell transplant who received donor lymphocyte infusions (DLI) engineered with HSV-TK for the purposes of treating disease relapse. The overall incidence of GVHD was 22% and the subsequent administration of ganciclovir controlled GVHD in all cases (199)

(200). This represents a very effective strategy for the control of GVHD post DLI, although the immunogenicity of HSV-TK leads to reduced persistence of the HSV-TK expressing cells (201).

An alternative suicide gene strategy is based on human apoptosis proteins and thus may be less immunogenic. A fusion protein consisting of a late stage apoptosis molecule, caspase 9 fused to a FK506 binding protein analogue has been used to transduce T lymphocytes. Apoptosis of the transduced cells is rapidly induced following administration of a chemical inducer of dimerization (CID) which results in aggregation and activation of caspase 9. In a study following patients treated with a haploidentical stem cell transplantation, 5 patients received donor T lymphocytes that had been transduced with the inducible caspase 9 construct. The DLI was administered to enhance immune reconstitution but carries a high risk of developing GVHD post transfer. 4/5 patients developed GVHD but the administration of a single dose of the non toxic CID drug led to elimination of >90% of the modified donor T cells within 30 minutes of administration and this led to eradication of GVHD in all patients without any later evidence of recurrence (202).

An additional safety concern of gene therapy is insertional mutagenesis secondary to insertion of the transgene into the host chromosome leading to disruption or aberrant activation of cellular genes. 19 X-linked severe combined immunodeficiency patients received haematopoietic stem cells transduced with retroviral vectors carrying the IL-2 receptor gamma (IL-2R- γ) chain and 5 patients have subsequently developed T cell leukaemia (203)

(204) (205). In 4 of the 5 cases the leukaemia arose secondary to retroviral integration in the region of a T cell oncogene, LMO2, resulting in its deregulated expression. There may also have been a potential role for the IL-2R- γ chain acting as a cooperating oncogene although this is controversial (206) (207) (208).

Both lentiviral and retroviral vectors integrate into the host genome in a semi random manner but tend to insert close to transcriptional units. Retroviral vectors integrate close to transcription start sites or DNA regulatory areas. Insertion at these points have a higher probability of causing deregulated gene expression (209) (210). Lentiviral vectors have a safer insertion profile, integrating within active transcription units (211) (212). Analysis of vector insertion hot spots within transduced haematopoietic stem cells showed that retroviral vector insertion sites were enriched at proto-oncogenes or genes controlling cell division but this was not seen with lentiviral vectors (213). Lentiviral vectors required a 10 fold higher rate of insertion events to induce cellular transformation compared to a retroviral vector with an LTR of comparable strength when used in a tumour prone mouse model (214).

Whilst the predominant mechanism of insertional mutagenesis produced by retroviral vectors is enhancer mediated host gene activation, an alternative mechanism has been demonstrated using lentiviral vectors. Chimeric fusion transcripts may arise containing both vector sequences and cellular mRNA due to alternative splicing (215) (216). Splicing from a donor site within the lentiviral vector to an adjacent gene can produce a novel coding fusion

transcript or splicing to an acceptor site within the vector can lead to a truncated cellular transcript leading to dysregulated protein turnover or translation. Lentiviral vectors in particular have the potential to carry multiple splice donor and acceptor sites as the unspliced viral genomic RNA is exported from the nucleus by the lentiviral protein, rev. In a clinical trial for treatment of beta thalassaemia using a lentiviral vector carrying the human β -globin gene, one of the transplanted patients developed a dominant myeloid clone that contained a integrated vector copy within the HMGA2 gene. The vector integration led to fusion of the splice donor sequence of HMGA2 with a cryptic splice acceptor site within an insulator element that had been inserted within the lentiviral LTR. This aberrant splicing led to premature termination of the HMGA2 transcript which had increased stability leading to abnormally high levels of the HMGA2 protein expression and this was thought to be the mechanism leading to clonal dominance in this patient (217).

Mature T cells appear to be less susceptible to cellular transformation arising from insertional mutagenesis than more primitive haematopoietic stem cells (HSC) which already have intrinsic self renewal activity. In addition to deregulation of gene expression by retroviral integration, the development of malignancy may also be dependent on transducing a cell type with intrinsic self renewal potential. The semi random integration of retroviral vectors in the genome of long term repopulating HSC may increase their self renewal potential or lead to malignant transformation. To assess the contribution of cell intrinsic features, Kustikova et al sorted HSC into the most primitive HSC and more mature haematopoietic progenitor cells prior to transduction with

retroviral vectors and transplanted into myeloablated syngeneic recipients. The clonal repertoire within the transplanted mice was analyzed and it was seen that clonal dominance only developed within the recipients of the most primitive self renewing HSCs which had a high frequency of insertional upregulation of proto-oncogenes. In contrast, insertional mutagenesis did not lead to clonal dominance following transplantation of more mature progenitor cells (218). T cells and more mature progenitor cells appear to be more resistant to the formation of expanding cell clones with oncogenic lesions. Newrzela et al compared transduced populations of mature T cells and HSC that had been transduced with identical high copy number retroviral vectors that encoded potent T cell oncogenes. After transplantation into RAG deficient mice, the mice that received transplantation of transduced HSC developed T cell lymphoma/leukaemia which had a characteristic phenotype and developed after a reproducible latency period. In contrast, none of the recipient mice that received mature T cells modified with the identical retroviral vector developed T cell lymphoma/leukemia despite the long term persistence of the gene modified T cells (219). Thus even in the context of multiple retroviral integrations with continuous high expression of a potent oncogene, on long term follow up the mature T cells remained untransformable. Follow up of patients who had received HSV-TK transduced T cells showed that up to 20% of gene insertions led to altered gene expression in the adjacent genes but this did not lead to transformation in any patients (220). No patients who have received adoptive immunotherapy of transduced T cells have developed malignancy as a direct result of the gene modified cells.

1.4.8 Chimeric antigen receptors

Another strategy to redirect T cell antigen specificity by gene transfer is to introduce chimeric antigen receptors (CAR). CAR consist of an antigen binding domain composed of an antibody derived single chain variable fragment (scFV) fused to an intracellular signaling domain (221). The advantages over TCR gene therapy is that CAR are non-HLA restricted and thus can be used in patients of any HLA type and also will not be affected by tumour down regulation of HLA molecules. In addition there is no risk of mispairing that arises with TCR gene therapy. The antigen targeted by the CAR must be expressed on the cell surface but they can be targeted to non protein surface molecules such as carbohydrates or glycolipids in addition to protein antigens. The first generation of CAR contained a CD3 ζ signaling domain and T cells modified with these CAR were able to secrete cytokines and lyse target cells following binding to specific antigen (222) (223) however they have shown limited clinical benefit due to ineffective activation or impaired persistence (224) (225) (226). The second generation of CAR incorporated the signaling domains of CD28 in addition to CD3 ζ which acts to enhance T cell proliferation upon ligation resulting in improved effector activity (227) (228) (229). This bypasses the need for the presence of a co-stimulatory ligand and thus prevents the induction of anergy or apoptosis in the absence of signal 2. Third generation CAR contain 2 co-stimulatory signaling domains such as 41BB or OX40 in addition to CD28 and have enhanced anti-tumour activity in vivo and improved persistence thought to be due to upregulation of antiapoptotic proteins like Bcl-xl (230).

1.5 THE ROLE OF CD4+ T CELLS IN ADOPTIVE IMMUNOTHERAPY

1.5.1 CD4+ T cell subsets

It is now well recognized that naïve CD4 T cells differentiate into a number of differing CD4+ T cell subsets each of which have a distinct immunological role and produce distinct cytokine profiles. Each subset also expresses lineage defining transcription factors that regulate cytokine production and effector function. There is increasing recognition that individual subsets of CD4+ T cells are not fixed lineages and show some degree of plasticity.

The Th1 and Th2 subsets of CD4+ T cells were first described in 1986 (231). Th1 cells are characterized by the production of IFN- γ and Lymphotoxin- α and the expression of the transcription factor, T-bet. Th1 cells are important mediators of immunity to intracellular pathogens and tumour cells via activation of macrophages and production of pro-inflammatory cytokines. Th2 cells are characterized by production of IL-4, IL-5 and IL-13. Their main role is the induction of IgE production by B cells, eosinophil activation and the eradication of extracellular parasites. GATA3 is the transcription factor expressed by Th2 cells (232) (233). Deregulated Th1 and Th2 responses play a role in certain diseases with Th1 cells being involved in many organ specific autoimmune diseases and Th2 cells promoting the development of allergy and atopy. In vitro, Th2 cells can be generated following T cell activation in the presence of IL-4 and IL-2 (234) (235) whilst Th1 cells can be generated in vitro in the presence of IL-12 and IFN- γ (236). The effector

cytokines that are produced by Th1 and Th2 cells act in a positive feedback loop to further amplify production of other Th1 cells or Th2 cells. They also act to repress the differentiation of the alternative subset explaining why development of Th1 and Th2 cells was believed to be mutually exclusive. The sequential activation of STAT1 by IFN- γ and STAT4 by IL-12 drives the optimal expression of T-bet (the central transcription factor for Th1 programming) whilst the activation of STAT6 by IL-4 upregulates GATA3. The stability of these developmental programs is enforced in part through the production by mature effectors of autocrine cytokine feedback loops.

The discovery of Th17 and T regulatory cells (Tregs) further expanded this Th1/Th2 paradigm. Th17 cells produce IL-17 and IL-23. They are characterized by the expression of transcription factors ROR- γ t and ROR α . Th17 cells can be generated from naïve CD4⁺ T cells by activation in the presence of TFG- β and IL-6 and are expanded in the presence of IL-23 (237) (238) (239). They have a role in eradication of certain extracellular bacteria and fungi, may play a role in anti tumour immunity, and are involved in inflammatory responses and the activation of innate immunity. Their role appears to overlap to some degree with that of Th1 cells. They have been shown to play a causative role in some autoimmune diseases that previously had been attributed to Th1 cells. Cytokines that induce Th1 and Th2 differentiation have been shown to be potent inhibitors of Th17 differentiation (240). IL-2 in particular has reciprocal effects on the production of Th17 cells and Tregs, acting to promote Treg differentiation whilst inhibiting Th17 differentiation (241) (242).

CD4⁺ Tregs have an essential immunosuppressive function acting to maintain peripheral tolerance and modulate immune responses. In vivo, Tregs exist in two forms. The naturally occurring Tregs (nTregs) develop within the thymus (243) whilst inducible Tregs (iTregs) can be generated in the periphery from naïve CD4⁺ T cells following T cell activation in the presence of IL-2 and TGF- β (244). Tregs constitutively express high levels of the IL-2R α chain (CD25) and they make up 5-10% of the peripheral CD4⁺ T cell population. Both T regulatory populations are characterized by the expression of the transcription factor, FOXP3 (245) (246). FOXP3 acts as the 'master regulator' for development and function of Tregs (245). Mice deficient in FOXP3 develop lethal autoimmune lymphoproliferative disease associated with a defect of CD4⁺ CD25⁺ Tregs (245).

One of the newest subsets of CD4⁺ T cells recently characterized are the T follicular helper CD4⁺ T cells (Tfh). Tfh cells are found within the germinal centres of lymphoid organs where they promote immunoglobulin class switching and somatic hypermutation in B cells. They produce IL-21 and express the transcription factor, Bcl-6 (247). Tfh are specialized in providing help for B cells within germinal centres. Differentiation of this population is driven by Bcl-6 which promotes entry of Tfh into germinal centres.

With the discovery of more populations of CD4⁺ T helper subsets, questions have arisen regarding the stability of these populations. When Th1 and Th2 cells were first described these were thought to represent stable and

committed lineages of cells. Each individual subset has been clearly characterized by the production of signature cytokines and for expression of lineage defining transcription factors. It has, however, recently been shown that there is a degree of plasticity. Whilst certain cytokines are uniquely produced by different subsets, other cytokines are expressed by a number of differing subsets. In addition, individual cells within the same T helper subset may demonstrate different patterns of cytokine production (e.g. within the Th1 subset, individual cells may be able to produce differing combinations of IFN- γ , LT α , IL-2 and TNF α). Only a very limited population will have the capacity to produce all of these cytokines simultaneously (248). There are now a number of examples of CD4⁺ T cell subsets that have flexible and plastic cytokine production. Th17 cells have been shown to acquire the ability to produce IFN- γ in addition to IL-17 (249) or to become selective producers of IFN- γ (250). This is in response to both IL-12 and IL-23 which leads to the induction of STAT4 and T-bet, both key transcription factors in the development of the Th1 phenotype. It has been postulated that Th1 cells may represent a terminally differentiated effector population with Th17 being the precursor lineage. GATA3⁺ IL-4⁺ Th2 cells can also be reprogrammed to also express T-bet and IFN- γ in the context of LCMV infection. These double positive populations were found to be crucial for virus eradication (251). Tfh appear to be the most fluid population of CD4⁺ T cells and have been shown to acquire attributes of Th1, Th2 or Th17 cells (252). With regards to Tregs, the loss of expression of FOXP3 is not infrequent. Tregs have also been shown to secrete IL-17 or IFN- γ and occasionally develop a pro-inflammatory

phenotype (253). The ability to produce IFN- γ does not always lead to loss of suppressive function.

Plasticity and the ability to switch phenotype in response to differing environmental and antigenic cues appears to be a key feature of CD4⁺ T cells. The expression of one lineage defining transcription factor was initially thought to generate a fixed cell phenotype, and the expression of key transcription factors was considered likely to be mutually exclusive e.g. T-bet in Th1 cells and GATA3 in Th2 cells. This is, however, overly simplistic, as it is now clear that these transcription factors are not expressed in such a restricted fashion. Individual CD4⁺ T cell subsets are much more heterogeneous than originally thought and much more dynamic. Moreover expression of more than one master regulator is a frequent event. Thus rather than a simplistic model of distinct expression of a single master regulator in terminally differentiated cells, it may be more accurate to think of co-expression of master regulators. These factors can be dynamically regulated by extrinsic and intrinsic factors and can fine tune T cell capabilities as the situation requires.

Epigenetic modifications are an important further modifier of CD4⁺ T cell differentiation and detailed epigenetic profiling has lent support to the concept of plasticity of CD4⁺ T cell subsets. Relaxation or condensation of the chromatin structure can lead to activation or repression of transcription respectively. Within a particular CD4⁺ T cell e.g. a Th1 cell, the lineage defining cytokine genes will be in a permissive configuration whilst the lineage

defining cytokine genes of the alternative CD4⁺ subtypes will be held in a repressive configuration. These findings suggested that the cell fate of differing CD4⁺ T cell subsets was fixed, with the signature cytokines of alternative cell fates being permanently switched off. However within a specified CD4⁺ subset e.g. Th1 cells, the genes encoding the transcription factors important for alternative cell fates e.g. GATA3, are not found in repressive configurations as might be expected. Instead the chromatin of such genes was found to be in both permissive and repressive configurations (252). This bivalent chromatin configuration ensures that the genes encoding master regulator transcription factors are not permanently switched off, allowing the cells to switch phenotype (254).

The inherent flexibility of CD4⁺ T cells and the ability to change phenotype in response to environmental and antigenic stimuli has clear implications for adoptive immunotherapy utilizing CD4⁺ T cells.

1.5.2 CD4⁺ T cells in tumour immunity

Both animal models and data from patients have enhanced the understanding of the role of CD4⁺ T cells in tumour immunity. CD4⁺ T cells can eradicate tumours in the absence of CD8⁺ T cells, and can provide 'help' for cytotoxic tumour specific CD8⁺ T cells. Greenberg et al used a murine model of disseminated acute leukaemia where recipient mice had been depleted of all CD8⁺ and CD4⁺ T cells. Adoptive transfer of leukaemia specific CD4⁺ T cells led to complete disease eradication of the MHC class II negative leukaemia

cells (255) even in the absence of cytotoxic CD8⁺ T cells. However, in the absence of CD4⁺ T cells, tumour control was lost or reduced. In a murine model of melanoma, mice were vaccinated with irradiated melanoma cells and then two weeks later re-challenged with unirradiated melanoma cells. Wild type mice rejected the tumour whilst CD4^{-/-} recipients had progressive tumour growth. Whilst CD8 knockout mice also had impaired ability to reject tumours, a significant proportion of CD8^{-/-} mice were still able to reject tumour (256).

Tumour specific CD4⁺ T cells have been isolated from patients with a wide variety of different tumour histologies. In patients with breast cancer, a high number of CD4⁺ T cells within the draining lymph nodes have been shown to be predictive for improved disease free survival (257) whilst in lung and hepatic carcinoma, the finding of high CD4/CD8 ratios correlated with improved disease free survival (258). In a phase II clinical trial using partially HLA matched allogeneic EBV specific cytotoxic T lymphocytes (CTLs) for treatment of EBV-positive post transplantation lymphoproliferative disorder (PTLD), it was shown that a higher percentage of CD4⁺ T cells within the infused CTLs was associated with a statistically significant improvement in overall response. In patients receiving CTLs that had <1% CD4⁺ T cells the overall response rate was 25% compared to 92% for those receiving a CTL infusion comprising >5% CD4⁺ T cells (259). Clinical trials in metastatic melanoma patients have shown that those who received adoptively transferred tumour specific CD8⁺ T cells alone had inferior responses to those receiving both tumour specific CD8⁺ and CD4⁺ T cells (260). For the

treatment of CMV reactivation post allogeneic bone marrow transplant, the adoptive transfer of CMV specific CD8+ T cells in isolation has been shown to require a 1000 fold higher dose of T cells than if given concurrently with CMV specific CD4+ T cells (261). Adoptive transfer of autologous tumour specific CD4+ T cells in a patient with metastatic melanoma (in the absence of lymphodepleting chemotherapy or IL-2) led to complete tumour regression with the patient remaining in complete remission 2 years post treatment. The adoptively transferred cells expanded 5000 fold following transfer and persisted long term. It was also noted that de novo CD8+ T cell clones reactive against melanoma antigens were generated (262).

1.5.3 Mechanism of action of tumour specific CD4+ T cells

CD4+ T cells are likely to have a central role in tumour eradication. They act to orchestrate the function of a wide variety of both innate and adaptive immune cells which have key roles in tumour eradication. CD4+ T cells play a role in the activation of tumour specific CD8+ T cells, the activation of APCs, macrophages and NK cells, whilst CD4+ T cells themselves can be directly cytotoxic.

The priming of naïve CD8+ CTLs requires the interaction of CD8+ and CD4+ T cells and the APC, with CD4+ T cells providing help via CD40L-CD40 signaling to the APC. Presentation of antigen within a non inflammatory environment e.g. in the tumour microenvironment, CD4+ T cell mediated help is provided via licensing of professional APCs. A number of models of

priming of CD4⁺ T cell dependent CTL responses demonstrated that in CD4 depleted or deficient mice, an activating CD40 antibody could efficiently restore antigen specific CD8⁺ T cell responses (263) (264) (265). Conversely, blockade of CD40L was observed to abrogate priming which was restored by adding back an activating CD40 antibody. The current model suggests that CD40L on CD4⁺ T cells binds to CD40 on professional APCs, which 'licenses' the APC, leading to upregulation of co-stimulatory molecules and cytokine secretion. The licensed APC then goes on to activate the CD8⁺ T cell via cross presentation of tumour antigen on class I MHC. A small proportion of CD8⁺ T cells may also express CD40 and it is possible that CD40L on CD4⁺ T cells can bind to CD40 on CD8⁺ T cells directly, but this model still requires presentation of antigen by an APC to the CD8⁺ T cell.

Perez Diaz compared tumour protection provided by monoclonal populations of tumour specific CD4⁺ and CD8⁺ T cells derived from anti-HY TCR transgenic mice. Tumour challenge experiments were carried out in Matahari TCR-transgenic mice where the CD8⁺ T cells are specific for HY-antigen, Uty presented by H2-D^b or in Marilyn TCR transgenic mice where the CD4⁺ T cells were specific for Dby presented by A^b. Mice were challenged with a C57BL/6 male bladder tumour which expressed both Uty and Dby antigens. The Matahari transgenic mice (CD8⁺) were not able to clear tumour whilst 80% of the Marilyn transgenic mice (CD4⁺) were able to mediate tumour rejection. Class II expression on the host tissues was essential for tumour rejection by CD4⁺ T cells suggesting that the CD4⁺ T cells were acting

indirectly following interaction with host APC and complete tumour clearance was dependent also on the presence of NK cells (266).

Corthay et al used a transgenic TCR system where T cells were specific for an idiotypic peptide from a variable region of an immunoglobulin light chain secreted by a myeloma cell line, MOPC315, presented by MHC II, I-E^d (267). Administration of syngeneic myeloma cells resulted in tumour rejection in transgenic mice where there was a high frequency of tumour specific CD4⁺ T cells but in non-transgenic mice there was fatal tumour growth. Tumour protection was dependent on recognition of the idiotype peptide and was CD4⁺ T cell mediated. The presence of B cells, $\gamma\delta$ T cells or CD8⁺ T cells was not required. The MOPC315 does not express or upregulate MHC II and could therefore not be directly recognized by the transgenic CD4⁺ T cells. CD4⁺ T cells in this system secreted IFN- γ which activated tumour infiltrating macrophages to kill the class II negative myeloma cells. A later study by the same group has also suggested a role for the anti-angiogenic chemokines CXCL9 and CXCL10 produced by tumour infiltrating macrophages in response to IFN- γ , which may halt tumour progression (268).

Quezada et al dissected the mechanisms of tumour protection using adoptive transfer of CD4⁺ TRP1⁺ (a melanoma differentiation antigen) transgenic T cells in a murine model of advanced melanoma (269). Transfer of only 50,000 naïve tumour reactive CD4⁺ TRP1⁺ T cells into irradiated recipients led to marked expansion and differentiation in vivo resulting in initial tumour regression in large established tumours. Approximately 60% of mice later

developed tumour recurrence. The addition of a blocking anti-CTLA4 antibody at the time of adoptive transfer of CD4⁺ T cells led to complete and persistent tumour eradication. The use of anti CTLA-4 led to an increased expansion of effector CD4⁺ TRP1⁺ T cells and a reduction in the number of CD4⁺ TRP1⁺ FOXP3⁺ T cells and the endogenous Treg population. Adoptively transferred CD4⁺ T cells had a Th1 phenotype, producing large amounts of TNF- α , IFN- γ and IL2 and again this was enhanced in the presence of anti CTLA-4. Tumor reactive CD4⁺ T cells expressed high levels of granzyme and showed in vivo cytotoxicity. Tumour rejection was dependent on production of IFN- γ : Neutralization of IFN- γ with a blocking antibody prevented tumour rejection. However, complete tumour eradication was still demonstrated in IFN- γ ^{-/-} recipient mice, suggesting that the adoptively transferred CD4⁺ TRP1⁺ T cells were producing IFN- γ and not host immune cells. IFN- γ receptor knock out mice also rejected established tumour, suggesting that the tumour cell itself was the direct target of the anti tumour effects of IFN- γ . Using RAG ^{-/-} recipient mice and perforin ^{-/-} mice it was shown that tumour rejection was also independent of the endogenous CD4, CD8 and B cell populations and of endogenous perforin killing activity. Depletion in vivo of NK1.1 cells and CD8⁺ T cells also failed to prevent tumour rejection. It was shown that adoptively transferred CD4⁺ TRP1⁺ cells were cytotoxic in vivo via direct recognition of class II MHC and this was mediated via degranulation of granzyme containing lytic granules. The tumour rejection was dependent on the expression of class II MHC by the tumour cells which was upregulated in response to IFN- γ production by the adoptively transferred CD4⁺ TRP1⁺ T cells.

CD4⁺ T cells also play a key role in generating CD8⁺ T cell memory responses. When CD8⁺ T cells encounter antigen they start to rapidly proliferate and during this time will also differentiate into distinct effector phenotypes and migrate to sites of infection or inflammation to eradicate pathogens. There is massive clonal expansion of antigen specific CD8⁺ T cells following antigen encounter but the vast majority of effector CD8⁺ T cells will die by apoptosis following clearance of pathogen leaving only about 10% of antigen specific CD8⁺ T cells to form the CD8⁺ T cell memory pool (270) (271) (272). CD4⁺ T cell mediated help is required at time of priming in order to generate robust CD8⁺ T cell memory responses and also maintains the CD8⁺ memory T cell pool. 2 models of generation of CD8⁺ memory T cells have been proposed, programming and maintenance, however this remains controversial and is probably dependent on the type of antigenic stimulus being studied. The programming model suggests that CD8⁺ memory T cells are programmed by CD4⁺ T cells during the first few days of initial activation. Subsequent to this, CD4⁺ T cell mediated help is not required for CD8⁺ T cell memory responses. Shedlock et al showed that CD8⁺ memory T cells that had been generated in CD4⁺ T cell replete mice could make normal recall responses if transferred into CD4⁺ T cell deplete hosts. However CD8⁺ memory T cells that had been generated in CD4⁺ T cell deficient hosts mounted defective memory responses even if transferred into CD4⁺ T cell replete hosts (273). The second model, maintenance, argues that CD4⁺ T cell mediated help is required during the entire phase of memory CD8⁺ T cell differentiation and is important not just for the generation of CD8⁺ memory T cells but also for maintenance of this population. CD8⁺ memory T cells that

have been generated in a wild type host decrease in number following transfer into a CD4⁺ T cell deficient host and showed functional impairment (274). The transfer of “unhelped” CD8⁺ T cells into a CD4⁺ T cell replete mouse model could actually lead to restoration of the impaired memory response suggesting that in this system, CD4⁺ T cell mediated help was not essential at priming.

Lack of CD4⁺ T cell mediated help at priming leads to upregulation of TNF-related apoptosis inducing ligand (TRAIL) upon secondary antigen encounter leading to antigen induced cell death (AICD) and diminished secondary responses. If CD4⁺ T cell mediated help is present it acts to alter the transcriptional program of the CD8⁺ T cells preventing the upregulation of TRAIL leading to successful secondary expansion (275). It is therefore possible that blockade of TRAIL could rescue the secondary expansion of “helpless” CD8 memory cells.

The presence of IL-2 at the time of CD8⁺ T cell priming is important for generation of functional CD8⁺ memory T cell responses. T cells primed in the absence of IL-2 have an impaired ability to expand upon antigen reencounter although IL-2 was not required for the primary CD8⁺ T cell response (270). IL-2 may be produced by CD4⁺ T cells acting in a paracrine fashion or may be derived from the CD8⁺ T cells themselves, following recognition of peptide-MHC on a licensed APC (276). The source of IL-2 i.e. paracrine vs. autocrine may be dependent on the type of antigen and/or strength of the priming stimulus. The magnitude or duration of IL-2 may also influence the

functional differentiation of CD8⁺ T cells. Prolonged IL-2 signaling has been shown to promote the development of short lived effector cells.

1.5.4 The role of class I restricted CD4⁺ T cells in tumour immunotherapy

With increasing recognition of the need for antigen specific CD4⁺ T cells for use in adoptive immunotherapy protocols, methods have been developed to generate tumour-associated antigen specific CD4⁺ T cells. Only a limited number of class II binding tumour epitopes have been defined and as a result the number of class II restricted tumour specific TCR for use in TCR gene transfer is very small. The number of known tumour peptides presented by class I is much larger and there is a much wider array of class I restricted TCRs available for use in TCR gene transfer and tumour protection models. For translational purposes, it will prove difficult to match patients for both MHC class I and II alleles for the available target epitopes. Redirecting CD4⁺ T cells to recognize class I restricted epitopes using TCR gene therapy may be more beneficial than introducing class II restricted TCRs and has greater translational potential. Class I restricted CD4⁺ T cells will be able to recognize directly presented tumour antigens and CD4⁺ and CD8⁺ T cells both expressing the same class I restricted TCR could be adoptively transferred together. By introducing a large number of tumour specific CD4⁺ T cells then the scope for engaging cognate help is vastly improved.

It has been shown by a number of groups that the introduction of a MHC class I restricted TCR into CD4⁺ T cells can generate functional class I restricted CD4⁺ helper T cells both in vitro and in vivo. The functional antigen-specific response of CD4⁺ T cells expressing class I restricted TCRs to a large part depends on the TCR that is used. TCRs differ in their dependence on the CD8 co-receptor. In the case of CD8 dependent TCRs, the co-transduction of the CD8 co-receptor is often required for the CD4⁺ T cells to be fully functional. When a CD8 independent TCR is transduced then there is less or no need to co-transduce the CD8 co-receptor in order to produce functional CD4⁺ T cells.

Kessels et al transduced CD4⁺ T cells with the OT1-TCR and compared them to CD4⁺ T cells co-transduced with OT1-TCR plus CD8 $\alpha\beta$ (6). OT1-TCR/CD8 $\alpha\beta$ co-transduced CD4⁺ T cells produced IFN- γ in response to specific antigen whilst OT1-TCR CD4⁺ T cells that lacked CD8 did not produce cytokines in response to antigen. To explore further the contribution that the CD8 co-receptor made to the function of OT1-TCR CD4⁺ T cells, they generated mutants of the CD8 α chain. The intracellular domain of CD8 α was replaced with the intracellular domain of the CD4 co-receptor or the CD8 α intracellular domain was deleted completely. OT1-TCR CD4 cells transduced with either CD8 $\alpha\beta$ mutant co-receptor retained antigen specific IFN- γ and IL-2 responses. The intracellular domain of CD8 α therefore did not appear to be essential for full function of the OT1-TCR expressing CD4⁺ T cells. Following adoptive transfer into mice, OT1-TCR CD4⁺ CD8 $\alpha\beta$ ⁺ T cells expanded in vivo in response to specific antigen. They also provided help for OT1-TCR CD8⁺

T cells when adoptively transferred into MHC class II deficient mice that had been infected with an OVA expressing influenza strain. In mice that received no CD4⁺ T cell help, the endogenous CD8⁺ T cell response did not increase above background but there was a substantial CD8 response if OT1-TCR CD4⁺ T cells were co-transferred with the OT1-TCR CD8⁺ T cells. They postulated that the role of the co-receptor in these cells was dependent on lipid raft association via the β chain of CD8. They found that functions such as cytokine production, CD40L expression and the ability to license APCs in vitro and to proliferate in vivo could not be improved by the inclusion of the CD4 co-receptor intracellular signaling domain and in addition all could be provided in the absence of CD8 α signaling domain.

Morris et al produced class I restricted CD4⁺ T cells by transduction of the F5-TCR which is specific for the influenza peptide, NP presented by H2-D^b (4). The in vitro and in vivo experiments used an EL4 lymphoma cell line which was stably transfected with NP peptide. EL4-NP cells express H2-D^b but don't express class II MHC. Following in vitro stimulation with peptide loaded syngeneic dendritic cells, 10pm of peptide could induce IFN- γ production by F5-TCR CD8⁺ T cells but a 10 fold higher concentration of peptide (100pm) was required to trigger IFN- γ secretion in F5 CD4⁺ T cells. F5-TCR CD4⁺ T cells produced little IFN- γ in response to class II negative EL4-NP cells whereas purified F5-TCR CD8⁺ T cells mounted an efficient IFN- γ response. If trans co-stimulation was provided with addition of syngeneic dendritic cells then the IFN- γ response of CD4⁺ F5-TCR T cells could be rescued. This was not due to cross presentation of NP by the dendritic cells as allogeneic

dendritic cells could also rescue the IFN- γ response to EL4-NP cells. Despite poor production of IFN- γ by F5-TCR CD4⁺ T cells, these cells could produce IL-2 and proliferate in response to stimulation with EL4-NP cells alone.

F5-TCR CD4⁺ T cells provided help and tumour protection in vivo when administered with a previously established non protective dose of F5-TCR CD8⁺ T cells in mice bearing EL4-NP tumours. The tumour protection conferred by the CD4⁺ T cells in this system was dependent on the co-administration of CD8⁺ T cells as transfer of F5-TCR CD4⁺ T cells with an anti-CD8 blocking antibody resulted in progressive tumour growth. However, F5-TCR CD4⁺ T cells were shown to persist up to 90 days post transfer and could expand upon tumour rechallenge.

It is thought that when a class I restricted TCR is introduced in to a CD4⁺ T cell that it continues to behave as a CD4⁺ T cell and does not adopt a CD8⁺ phenotype. Expression of the CD4 and CD8 co-receptors on thymocytes affects their differentiation during development but it is not known whether the effector function in the periphery is affected by the interaction of the CD4 or CD8 co-receptor and whether the TCR is class I or II restricted. Does the introduced TCR dictate the polarization of the cell, or is the functional polarization an imprinted property acquired during development or is it acquired in culture conditions or altered by the introduced TCR?

1.6 CONCLUSION

TCR gene therapy allows the specificity of T cells to be redirected, producing tumour specific T cells that can be adoptively transferred in vivo to target and eradicate tumour cells. This strategy has the future potential to produce tumour specific T cells for all cancer patients for use in adoptive immunotherapy and to generate tumour specific T cells with greatly enhanced function. Initial clinical studies have shown the potential benefits of TCR gene therapy but refinements are ongoing to improve the overall efficacy of this technique and also to improve the safety profile of TCR gene modified T cells. The tumour protection provided by TCR gene modified T cells is still inferior to that provided following the adoptive transfer of TIL. There are several reasons that could explain a functional impairment of TCR gene modified T cells compared to non-modified T cells. TCR chains must combine with CD3 molecules to form a complex prior to expression on the T cell surface. In the absence of CD3, TCRs don't assemble properly and are degraded. Gene modified T cells express endogenous and also introduced TCR chains and they must compete for a limited number of CD3 molecules. Competition may reduce cell surface expression of the introduced TCR, which may impair the avidity of antigen recognition. The incorrect pairing of introduced and endogenous TCR α/β chains could also reduce the avidity of transduced T cells. The introduction of an additional disulphide bond between the α and β chains, or sequence modifications in the TCR constant regions can reduce the mis-pairing between the introduced and endogenous TCR chains and improve TCR expression in transduced T cells.

Finally, current TCR gene transfer protocols frequently produce functional CD8⁺ T cells, but not CD4⁺ T cells. The adoptive transfer of antigen-specific CD8⁺ T cells in the absence of antigen-specific CD4⁺ T cells is likely to impair tumour protection. This project focuses on improving the efficacy of CD4⁺ T cells that have been modified to express a class I restricted TCR by co-transducing TCR in conjunction with additional CD3 molecules. With this strategy, the aim is to increase the surface expression of the transduced class I restricted TCR in CD4⁺ T cells and that by this mechanism, the in vitro and in vivo functional avidity of the class I restricted CD4⁺ T cell can be improved. By improving the in vivo function of these transduced CD4⁺ T cells, it has been hypothesized that this will translate to more effective anti-tumour responses following adoptive transfer.

1.7 AIMS AND OBJECTIVES

In this project I explored whether the co-transfer of CD3 genes can improve the function of MHC class I-restricted TCRs in CD4⁺ helper T cells. This strategy utilized murine tumour models to test whether TCR+CD3 gene transfer could enhance tumour protection by adoptively transferred CD4⁺ and CD8⁺ T cells without increasing on-target or off-target toxicity. The following working hypotheses were tested:

- 1) Co-transfer of TCR+CD3 enhances the functional activity of MHC-class I-restricted TCRs in CD4⁺ helper T cells.

- 2) Co-transfer of TCR+CD3 improves antigen-specific T cell function in vivo.
- 3) Adoptive transfer of TCR+CD3 modified T cells results in enhanced tumour protection in vivo.
- 4) Adoptive transfer of TCR+CD3 modified T cells does not result in on-target or off-target toxicity in vivo.

CHAPTER 2 - MATERIALS AND METHODS

2.1 MICE

Mice were obtained from an established breeding colony in our on site animal facility. For in vitro work, C57BL/6 female mice were used as splenocyte donors. For in vivo work, Thy 1.1+ C57BL/6 female mice aged 10-12 weeks were used as donors and Thy 1.2+ C57BL/6 female mice aged 10-12 weeks were used as recipients unless otherwise stated. For production of bone marrow chimeric mice, 10 week old female F1 (C57BL/6 x Balb/c) mice, 10 week old female Thy 1.1+ Balb/c mice and 10 week old Thy 1.1+ C57BL/6 female mice were utilized (described below in section 2.7.4). Thy 1.1+ C57BL/6 Luciferase+ transgenic mice were a kind gift from Doctor R Zeiser (Freiburg University, Germany). All work was carried out under a UK home office project license.

2.2 IN VITRO CELL CULTURE

2.2.1 Culture of Phoenix Ecotropic (Ph-Eco) packaging cell line

All tissue culture procedures were performed in a class II tissue culture cabinet. Ph-Eco adherent cells (Nolan Laboratory, Stanford, CA) were used as the packaging line for production of retroviral particles. Ph-Eco cells were cultured in T75 tissue culture flasks (Helena Biosciences, UK) in IMDM complete medium (IMDM medium) (Lonza) containing 10% heat inactivated

Foetal Calf Serum (FCS) (Biowest, France), 1% Penicillin/Streptomycin (Invitrogen) and 2mM L-glutamine (Sigma-Aldrich).

2.2.2 Culture of EL4 and EL4-NP tumour cell lines

EL4 cells (Sigma) are murine lymphoma cells derived from C57BL/6 mice. EL4-NP tumour cell lines are EL4 cells that have been stably transfected with influenza A nucleoprotein (NP) and were a kind gift from Doctor B Stockinger (National Institute for Medical Research, London). EL4 and EL4-NP cells express H2-D^b but do not express class II MHC. EL4 and EL4-NP cells were cultured in suspension in T75 culture flasks in IMDM complete medium. EL4-NP Luciferase+ cells were produced by transfection of EL4-NP cells with a red shifted luciferase plasmid and were a kind gift from Doctor M Pule (University College London, London).

2.3 PEPTIDES

All peptides were obtained from Proimmune (Oxford, UK). The influenza virus A-NP derived synthetic peptide pNP366 (ASNENMDAM) binds to H2-D^b molecules. The Wilms' Tumour protein (WT1) derived peptide pWT126 (RMFPNAPYL) was used as an H2-D^b binding control. Peptides were reconstituted in PBS to a stock concentration of 2mM and stored at -20°C.

2.4 GENERATION OF TCR TRANSDUCED CELLS

2.4.1 Retroviral Vectors

The retroviral vectors pMP71-F5 α -2A-F5 β (F5 TCR), pMP71-CD3- ζ -2A- ϵ -2A- δ -2A- γ -IRES-GFP (CD3-GFP), pMP71-rWT1-IRES-GFP (GFP control) and pMP71-F5 β (F5 β chain) were used for transduction. The F5-TCR vector encodes the α and β chains of the F5-TCR with the α and β chains of the F5-TCR separated by a 2A sequence. The F5 TCR recognizes the influenza A virus NP (NP366-379) peptide in the context of murine D^b molecules. The CD3-GFP vector contains all four chains of the CD3 complex linked by 2A sequences and followed by an IRES-GFP sequence so that transduction of CD3-GFP vector can be assessed by GFP expression. The GFP-control vector has the WT1 gene inserted into the cloning site in reverse orientation so that only the GFP sequence is translated from this vector. The pMP71-F5 β -TCR vector contains only the β chain of the F5 TCR and production of this vector is described in Section 2.7. pCL-Eco is a retroviral packaging vector, which is used to enhance retroviral titres produced by Ph-Eco cells. (Vector maps of F5-TCR, CD3-GFP and F5-TCR β are shown in Figure 6 and 7 and sequences in Appendix 2.)

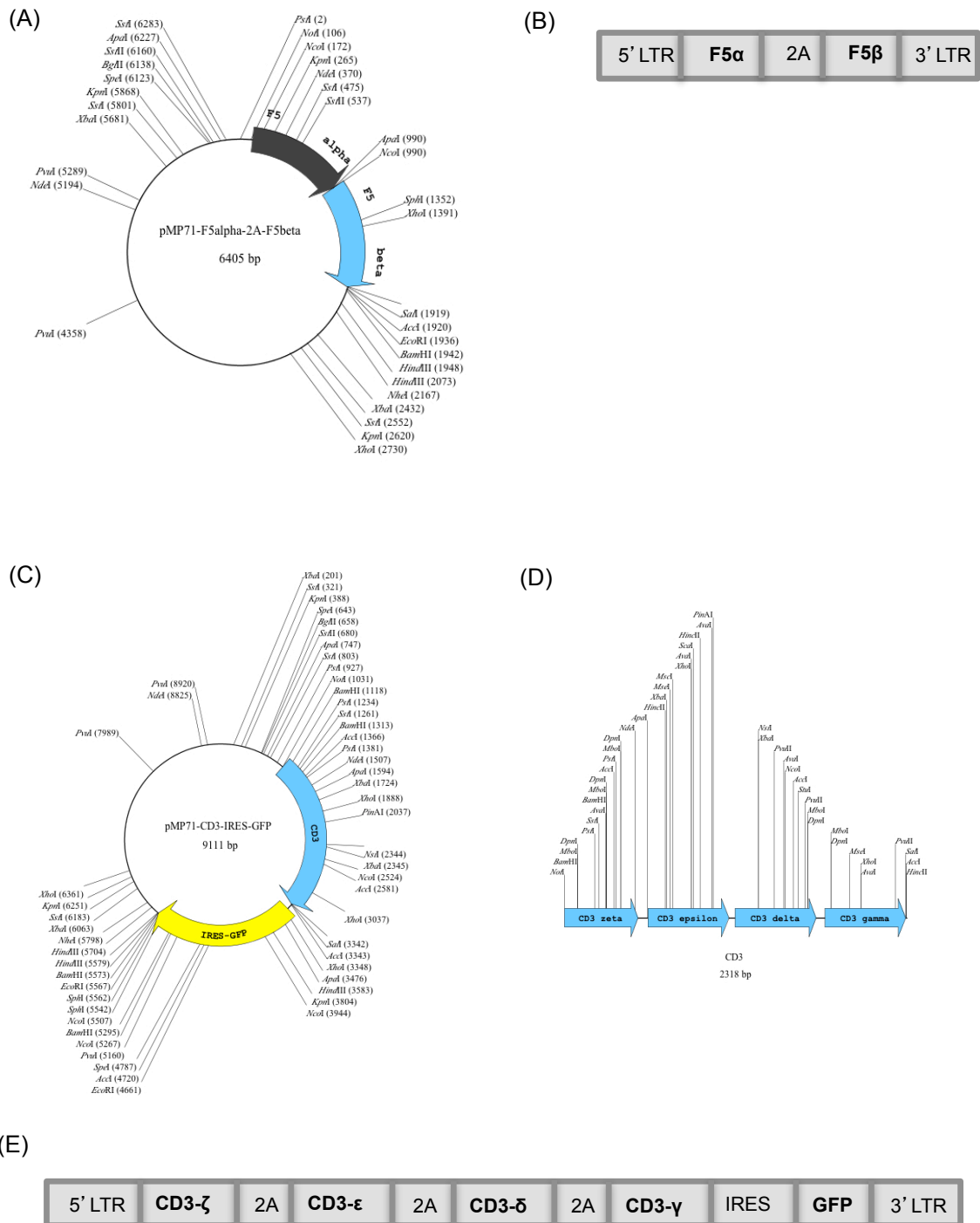
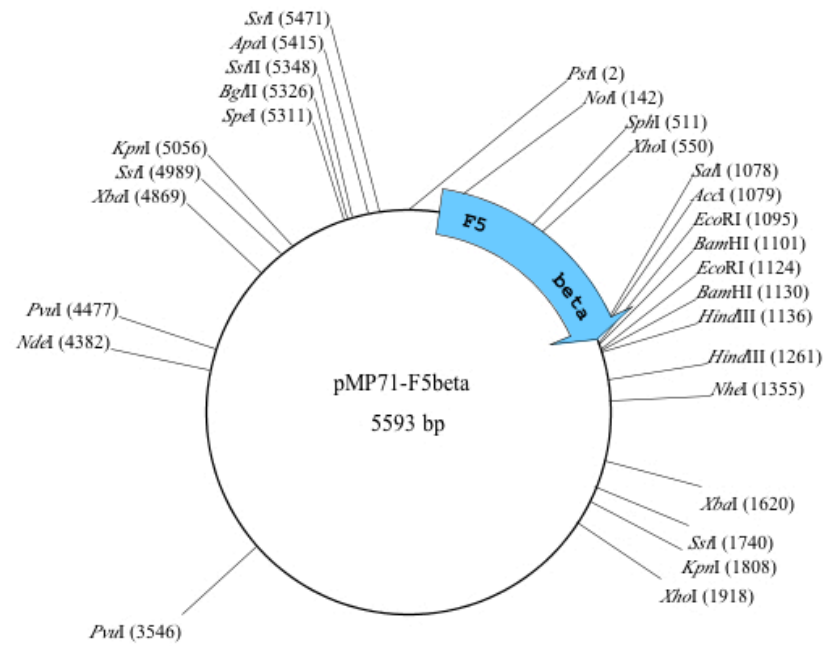


Figure 6 – pMP71 F5-TCR and pMP71 CD3-IRES-GFP retroviral vectors. (A) pMP71 F5-TCR Vector Map and (B) Schematic outline of F5-TCR insert. (C) pMP71 CD3-IRES-GFP Vector Map and (D) CD3 insert encoding zeta, epsilon, delta and gamma chains of CD3 complex, separated by 2A sequences. (E) Schematic outline of CD3-IRES-GFP insert.

(A)



(B)



Figure 7 –pMP71-F5-TCRβ retroviral vector. (A) pMP71-F5-TCRβ retroviral vector map (B) Schematic outline of F5-TCRβ insert.

2.4.2 Transfection of Ph-Eco cells

24 hours prior to transfection, 1.5×10^6 Ph-Eco cells were plated in 8ml of IMDM complete medium in 10 cm tissue culture plates (Nunc, Denmark).

Fugene (Roche) was used for transfection of Ph-Eco Cells. 10 μ l of Fugene was added to 300 μ l of Optimum buffer and incubated at room temperature for 5 minutes. A mixture of 2.6 μ g of retroviral vector DNA and 1.5 μ g of pCL-Eco was re-suspended in distilled water at a final volume of 50 μ l and then added to the Fugene/Optimum mix. Following a 15 minute incubation at room temperature, the transfection mix was added drop wise to the plated Ph-Eco cells. 24 hours following transfection, the medium was removed from the Ph-Eco cells and replaced with 5.5ml of RPMI complete medium (RPMI 1640 (Lonza), 10% heat inactivated FCS (Sigma), 1% Penicillin/Streptomycin, 1% 2mM L-glutamine and 0.5% 2-Mercaptoethanol (Sigma, UK). After a further 24 hours incubation, the retroviral supernatant was harvested and centrifuged at 1500rpm for 5 minutes to remove any cellular debris. The retroviral supernatant was then used directly for transduction of murine splenocytes

2.4.3 CD4+ T cell purification and activation

Splenocytes were obtained from C57BL/6 (H2-D^b) female mice. CD4+ splenocytes were obtained by positive selection using anti CD4 magnetic beads (Miltenyi) and an LS separation magnetic column (Miltenyi) as per manufacturer's protocol. CD4+ T cells were activated with CD3/CD28 Dynabeads (Invitrogen) and IL-2 (Roche) 30U/ml. CD4+ T cells were re-

suspended at 1×10^6 cells/ml in RPMI complete medium in 6 well tissue culture plate. CD4+ T cells were activated for 24 hours prior to transduction.

2.4.4 Retroviral Transduction

Non treated 6 well tissue culture plates (Cellstar) were coated with 2.5 ml retronectin/well (Takara Bio, Japan) and incubated at room temperature for 2 hours. Following coating with retronectin, plates were blocked for 30 minutes at room temperature with filter sterilized 2% Bovine Serum Albumin (BSA)/PBS (2.5ml per well). For transduction of F5 TCR alone, 4×10^6 activated splenocytes were re-suspended in 1.5 ml F5 TCR viral supernatant and 1.5ml RPMI complete medium and added to one well of a retronectin coated plate. For co-transduction of cells with F5 TCR and CD3-GFP, 4×10^6 activated splenocytes were re-suspended in 1.5ml F5 TCR viral supernatant and 1.5 mls CD3-GFP viral supernatant. For co-transduction of cells with F5 TCR and GFP-control, 4×10^6 activated splenocytes were re-suspended in 1.5ml F5 TCR viral supernatant and 1.5 mls GFP-control viral supernatant. For mock transduction, 4×10^6 activated splenocytes were re-suspended in 3 ml RPMI complete medium. Cells were centrifuged at 2000rpm for 90 minutes with no brake and then incubated at 37°C, 5% CO₂. On day 1 and day 3 post transduction, transduced splenocytes were re-suspended in 6ml fresh RPMI complete medium and fresh IL-2 (Chirion) was added at 100U/ml. CD3/CD28 activation beads were removed by applying transduced cells to a magnet on day 1 post transduction.

2.5 FLOW CYTOMETRY

FACS analysis to assess transduction efficiency was assessed from day 3 post transduction onwards. Samples were stained in FACS buffer (PBS and 1% FCS) with the appropriate dilution of relevant monoclonal antibodies. F5 TCR expression was assessed by staining with anti murine V β -11-PE or NP-Pentamer-PE (Pro-immune). Expression of CD3-GFP or GFP control was assessed by GFP expression. The following monoclonal antibodies were used for flow cytometric staining: rat anti-mouse CD4-APC; rat anti-mouse CD4 APC-H7; rat anti-mouse CD8 APC; rat anti-mouse CD8-V450; rat anti-mouse V β 11-PE; rat anti-mouse IFN- γ APC; rat anti-mouse Thy 1.1 PeCy7; rat anti-mouse CD19 PerCP-Cy5; rat anti-mouse H2-K^b-PE; rat anti-mouse H2-K^d-FITC; rat anti-mouse CD11b APC (All BD Biosciences) and rat anti-mouse IL-2-APC (eBiosciences). Samples were acquired on an LSR2 Flow Cytometer (BD Biosciences) and analyzed using FACS DIVA software or Flowjo (Treestar, Oregon). FACS sorting of transduced cells was carried out where indicated using a FACS Aria cell sorter. Transduced Cells were stained as per standard protocols prior to sort and re-suspended in RPMI supplemented with 1% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine, 0.1% Gentamicin, 5% Amphotericin, 2.5% HEPES buffer and 2mM EDTA. Post FACS sorting, cells were collected in medium as above but supplemented with 10% FCS.

2.6 IN VITRO FUNCTIONAL ASSAYS

2.6.1 IFN- γ and IL-2 ELISA and proliferation measured by Thymidine incorporation assay

On day 5 post transduction, transduced CD4⁺ T cells were re-stimulated with peptide loaded C57BL/6 splenocytes. 5-10 x 10⁶ stimulator cells were re-suspended in 1ml RPMI complete medium. Relevant peptide or irrelevant peptide were added at the appropriate concentration to stimulator cells and cells were then incubated for 1-2 hours at 37°C, 5% CO₂ and then irradiated with 40 Gy. Stimulator cells were loaded with NP peptide starting at a concentration of 10 μ M with a 1 in 10 peptide titration down to 1pM NP peptide. As a negative control C57BL/6 splenocytes were peptide loaded with 10 μ M irrelevant peptide (WT126 peptide).

Re-stimulation of transduced cells was carried out in 96 well round bottomed plates. 5 x 10⁴ transduced cells were incubated with 5 x 10⁴ peptide loaded stimulator cells in 200 μ l RPMI complete medium/well. Each condition was performed in triplicate. Cells were incubated for 24 hours at 37°C, 5% CO₂. Supernatant was then harvested for use in ELISA to measure IL-2 and IFN- γ production. ELISA assays were performed as per manufacturer's protocol using BD ELISA kits. For proliferation assays, cells were pulsed with 0.5 μ Ci (1Ci = 37GBq) {³H} thymidine and then incubated for a further 24 hours. The cells were then harvested using an automated 96 well harvester (Amersham

Pharmacia) and thymidine incorporation measured using a gamma counter (Wallace, UK).

2.6.2 Proliferation Assay using cell proliferation dye eFluor® 670

Cell proliferation dye eFluor 670® (eBioscience) was diluted in PBS to give a final concentration of 1µM. Transduced F5-TCR and F5-TCR + CD3 T cells were washed twice in PBS then each population of cells were re-suspended in 1ml of e-Fluor 670® and incubated at 37°C for 3 minutes. Cells were quenched with 4ml of 8% FCS-PBS (ice cold) and then centrifuged at 1500rpm for 5 minutes at 4°C. Cells were then re-suspended in 10ml 2% FCS-PBS (ice cold) and centrifuged at 1500rpm for 5 minutes, 4°C. This washing step was repeated twice. Labelled transduced T cells were re-suspended at 1×10^6 /ml in RPMI complete medium. 0.1×10^6 T cells were co-cultured with 0.1×10^6 peptide loaded splenocytes in a total volume of 200µl/well in a 96 well round bottomed plate. Cells were incubated for 48 hours at 37°C. Prior to FACS analysis, cells were labeled with CD8-PerCP or CD4-PerCP and Vβ11-PE.

2.6.3 Intracellular cytokine staining

Peptide loading of stimulator cells was carried out as described above in section 2.6.1. For intracellular cytokine staining, 3×10^5 transduced cells were incubated with 3×10^5 peptide loaded stimulator cells in 200µl RPMI complete medium/well of a 96 well round bottomed plate. PMA (25-50ng/ml final

concentration) and Ionomycin (0.5-1µg/ml final concentration) were added to 3×10^5 transduced cells in 200µl RPMI complete medium per well as a positive control. Cells were incubated for 2 hours and then Brefeldin A (Sigma) at 10µg/ml final concentration was added to all wells. Cells were then incubated at 37°C, 5% CO₂ for a further 16 hours prior to fixation and permeabilization steps.

Cells were stained with cell surface markers prior to fixation and permeabilization. Fixation and permeabilization was carried out as per standard protocols using BD Cytofix/Cytoperm™ kit. Cells were then stained with either IFN-γ-APC or IL-2-APC diluted at the appropriate concentration in 50µl BD Perm/Wash™. Cells were incubated for 30 minutes at 4°C in the dark. Cells were washed twice in 200µl in BD Perm/Wash and then re-suspended in 200µl FACS buffer prior to FACS analysis.

2.6.4 Stimulation with EL4 or EL4-NP tumour cells

On day 5 post transduction, transduced CD4+ T cells were stimulated with EL4 tumour cells or EL4-NP tumour cells – either alone or with bone marrow derived dendritic cells. Bone marrow derived dendritic cells (BMDCs) were prepared from C57BL/6 mice bone marrow cells. Bone marrow cells were harvested and cultured at 1×10^6 cells/ml in RPMI complete medium plus GM-CSF 10ng/ml. On day +5, LPS was added at 1µg/ml and dendritic cells were harvested and used for assay on day 6 post production. BMDCs were peptide loaded with 10µM NP or 10µM WT peptide or were left unloaded.

Following peptide loading BMDCs and tumour cells were irradiated prior to set up of overnight stimulation. Supernatant was collected 24 hours later for use in ELISA.

2.6.5 Cytokine production measured by luminex technology

Populations of F5-TCR CD4+, F5-TCR + CD3 CD4+, F5-TCR CD8+ and F5-TCR + CD3 CD8+ transduced T cell populations were stimulated overnight with peptide loaded splenocytes as described in Section 2.6.1. Supernatant was collected following 24 hours of stimulation and then used for multiple cytokine analysis using luminex technology as per the manufacturer's protocol (Invitrogen).

2.7 PRODUCTION OF PMP71-F5-TCR β RETROVIRAL VECTOR

The pMP71-F5-TCR β vector was generated from the original pMP71-F5-TCR vector. Primers were designed that amplified only the β chain region of the F5-TCR which had been engineered to introduce an additional Not1 restriction enzyme site at the 5' end of F5-TCR β , just prior to the start codon. The F5-TCR is ordinarily flanked by a Not1 and Sal 1 site but the Not1 site in the pMP71-F5-TCR vector is at the 5' end of F5-TCR α . The F5-TCR β chain was amplified using PCR with the following PCR mix: 1 μ l pMP71-F5-TCR DNA (10ng/ μ l); 25 μ l Phusion® High Fidelity PCR Mix (NEB); 1 μ l forward primer (0.5 μ M); 1 μ l reverse primer (0.5 μ M); 22 μ l distilled water. The reaction conditions were as follows: For 35 cycles: 98°C for 30 seconds; 98°C for 10

seconds; 72°C for 70 seconds followed by 72°C for 10 minutes. The resulting PCR products were run on a 1% agarose gel (Figure 8A). The PCR product was purified using a Qiagen PCR purification kit and then the product digested as follows for 1 hour at 37°C. PCR product 30µl; NEB buffer 3 35µl, BSA 0.5µl; Not 1 1.5µl (NEB); Sal1 1.5µl, distilled water 11.5µl. In order to obtain the pMP71 backbone the original pMP71-F5-TCR vector was digested for 1 hour at 37° as follows: pMP71-F5-TCR DNA 10µl (1µg/µl); NEB Buffer 3 5µl; Sal1 1.5µl (NEB); Not1 1.5µl (NEB), distilled water 32µl. The digestion products were run on a 1% agarose gel (Figure 8B). The pMP71 backbone was cut out from the agarose gel and then gel extraction carried out using a Qiagen gel extraction kit. Figure 8C shows the pMP71 backbone and F5-TCRβ insert just prior to ligation. The pMP71 backbone and F5-TCRβ were ligated together at room temperature for 15 minutes as follows: pMP71 backbone 3µl; F5-TCRβ insert 2µl; distilled water 6µl; ligase buffer 1µl; quick ligase (NEB) 1µl. Following ligation, max efficiency DH5α cells™ (Invitrogen) were transformed with the pMP71 vector as follows: 50µl of cells were defrosted and kept on ice for 5-10 minutes. 2µl of ligation mix was added to the cells and then incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 45 seconds and then incubated on ice for 2 minutes. 250µl of SOC buffer was added to the transformation mixture and then shaken at 37°C for 1 hour. 100µl of transformation mix was then spread thinly on agar plates and incubated overnight at 37°C. The following morning, colonies were picked from the plate and then miniprep of the plasmid

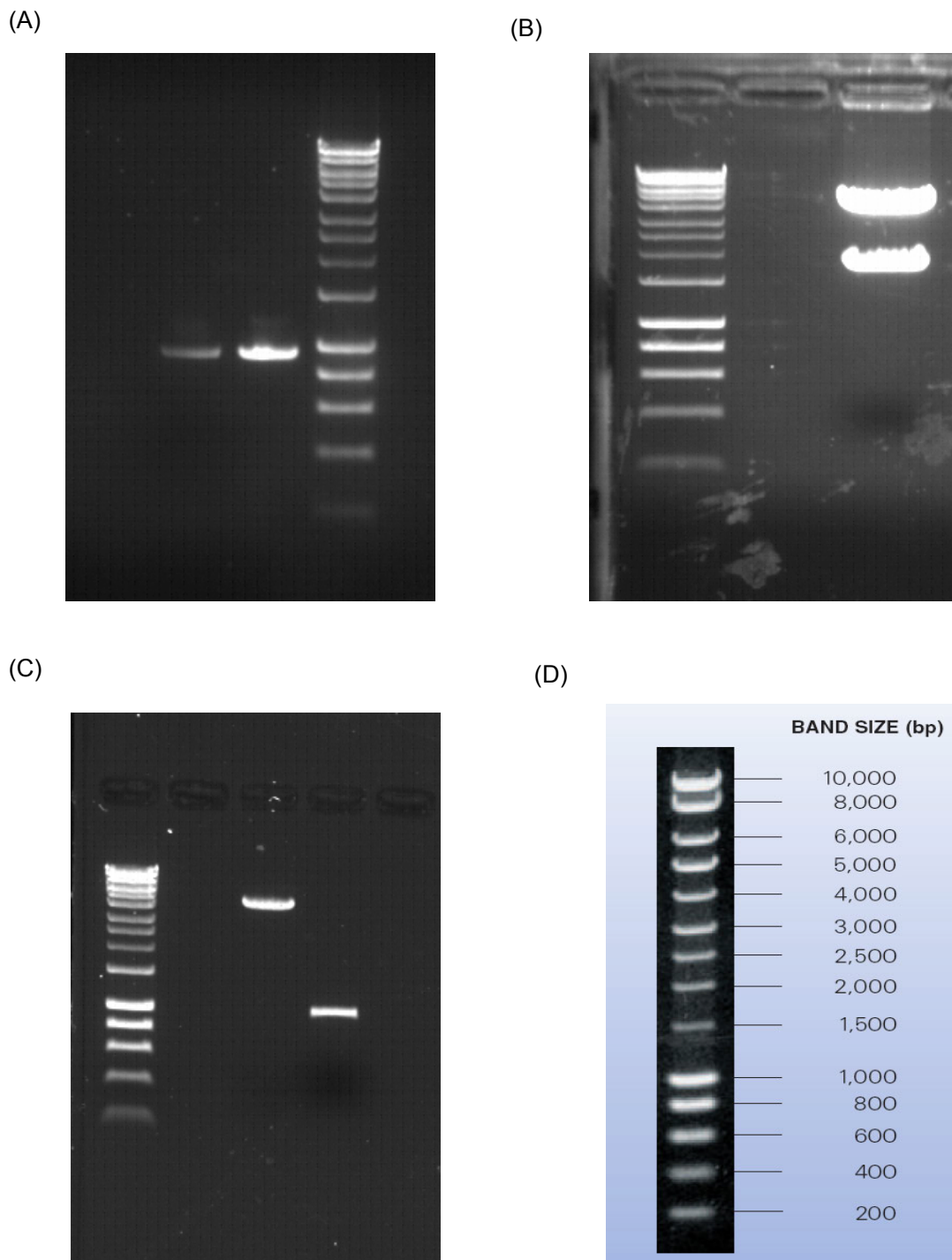


Figure 8 – Production of pMP71-F5-TCR β vector. Production of the pMP71-F5-TCR β vector is outlined in Chapter 2 Section 2.7. (A) PCR product of F5-TCR β insert (950bp) (B) Digestion products of pMP71-F5TCR showing the pMP71 backbone (4592bp) and the original F5 insert (1813bp). (C) Pre ligation gel showing pMP71 backbone and F5-TCR β insert prior to ligation (D) Hyper ladder 1 demonstrating approximate band size.

produced using a Qiagen miniprep kit. The miniprep DNA was then used to maxiprep the pMP71-F5-TCR β vector using a Qiagen kit. DNA was resuspended post maxiprep at 1 μ g/ μ l and was stored at -20°C. The pMP71-F5-TCR β vector was sequenced prior to use in TCR transduction experiments (Appendix 1).

2.8 ADOPTIVE TRANSFER OF F5 TCR AND F5 TCR-CD3 CD4+ T CELLS

2.8.1 Tumour challenge experimental set up

Splenocytes for transduction were obtained from C57BL/6 Thy1.1 mice. CD4+ T cells were transduced with F5 TCR or with F5 TCR and CD3-GFP as described in section 2.4. On day 2 post transduction, C57BL/6 Thy 1.2 recipient mice were irradiated with 5.5Gy. 4 hours post irradiation mice were injected with 1 x 10⁶ EL4-NP cells subcutaneously in the right flank. 1 x 10⁶ EL4-NP cells were re-suspended in 100 μ l final volume of 50:50 mix of PBS and Matrigel prior to injection. On day 3 post transduction, TCR transduced cells were injected intravenously via the tail vein at the appropriate cell dose as described in the results section. For some experiments, TCR transduced CD4+ T cells were FACS sorted prior to injection into purified populations of F5 TCR CD4+ cells and F5 TCR-CD3 CD4+ T cells (typically purity was >92% prior to injection). Mice were housed in pathogen free conditions in an IVC unit and received 5% Baytril (Bayer) for one week prior to irradiation and 2 weeks post. Tumour size was measured using calipers every other day and mice were sacrificed if tumour size exceeded 15mm in any diameter or

ulceration was observed at any size. Spleen, bone marrow, lymph nodes and peripheral blood were harvested for analysis on day of sacrifice. Single cell suspension of spleen, bone marrow and lymph nodes was prepared and red cell lysis carried out using ACK lysis buffer (Lonza) on spleen and bone marrow. Cells were counted and then stained for FACS analysis, which was carried out as in section 6.1.5. Cells were stained with Thy1.1-PE-Cy7, CD4-APC-H7, V β 11-PE, CD62L-APC and CD44 V450. Where indicated, for certain experiments a lower radiation dose of 4 Gy was used.

2.8.2 In vivo bioluminescence imaging of tumour

In certain tumour protection experiments, EL4-NP-luciferase positive tumour cells were used so that tumour growth could be followed using caliper measurement and bioluminescence imaging. Mice were injected subcutaneously with of 7.5 mg/kg D-luciferin firefly (Biosynth). Mice were anaesthetized 10 minutes post injection and imaged with a Xenogen IVIS-100 (Caliper Life Sciences).

2.8.3 In vivo bioluminescence imaging of transduced T cells

Splenocytes for transduction were obtained from C57BL/6 Thy1.1 luciferase+ mice. CD4+ T cells were transduced with F5 TCR or with F5 TCR and CD3-GFP as per methods section 2.4. On day 2 post transduction, C57BL/6 mice were irradiated with 5.5 Gy. 4 hours post irradiation mice were injected with 1×10^6 EL4-NP cells subcutaneously in the right flank. 1×10^6 EL4-NP cells

were re-suspended in 100µl final volume of 50:50 mix of PBS and Matrigel prior to injection. On day 3 post transduction (24 hours post irradiation), 1×10^6 TCR transduced cells in 200µl PBS were injected intravenously via the tail vein. Prior to injection, transduced cells had been CD8 depleted using anti-CD8 beads and LD depletion column (Miltenyi) to ensure that purified CD4⁺ T cells were administered. Mice were imaged on day 3, 5, 7 and 10 post injection. Mice were injected intraperitoneally with 15mg/Kg D-Luciferin Firefly (Biosynth). Mice were anaesthetized 10 minutes post injection and imaged with a Xenogen IVIS-100 (Caliper Life Sciences).

2.8.4 Production of chimeric mice

10 week old F1(C57BL/6 x Balb/c) mice were irradiated with 5.5Gy and then irradiated 48 hours later with a further 5.5Gy. T cell depleted donor bone marrow cells were obtained from Thy 1.1⁺ Balb/c mice or Thy 1.1⁺ C57BL/6 mice. Donor bone marrow was depleted of CD4⁺ and CD8⁺ T cells prior to injection using magnetic anti-CD4 and anti-CD8 beads (Miltenyi) and LD depletion column. F1 recipient mice were injected with 5×10^6 T cell depleted Balb/c or C57BL/6 bone marrow cells intravenously. Mice were housed in pathogen free conditions and checked for achievement of full donor chimerism at 12 weeks post original bone marrow transfer. To check full donor chimerism, peripheral blood samples were stained for expression of CD19, CD4, CD8, CD11b, H2-K^b and H2-K^d.

2.8.5 Tumour challenge experiment using chimeric F1 mice

{C57BL/6→F1} and {Balb/c→F1} bone marrow chimeric mice were used as recipients. Splenocytes for transduction were obtained from F1 (C57BL/6 x Balb/c) mice. CD4⁺ T cells were transduced with F5-TCR or with F5-TCR and CD3-GFP as per methods section 2.4. On day 2 post transduction, recipient {C57BL/6→F1} and {Balb/c→F1} bone marrow chimeric mice were irradiated with 4 Gy. 4 hours post irradiation mice were injected with 1×10^6 EL4-NP-luciferase cells subcutaneously in the right flank. On day 3 post transduction (20 hours post irradiation), 1×10^6 F5-TCR transduced cells in 200 μ l PBS were injected intravenously via the tail vein. Prior to injection, transduced cells had been CD8 depleted using anti-CD8 beads and a LD depletion column (Miltenyi) to ensure that purified CD4⁺ T cells were administered. Tumour size was monitored using calipers and by bioluminescence imaging as above. Mice were sacrificed if tumour size exceeded 15mm in any diameter or if severity score exceeded 3.5 or if >20% loss of weight from baseline. Spleen, bone marrow, lymph nodes and peripheral blood were harvested for analysis on day of takedown. Single cell suspension of spleen, bone marrow and lymph nodes was prepared and red cell lysis carried out using ACK lysis buffer (Lonza) on spleen and bone marrow. Cells were counted and then staining for FACS analysis was carried out as in section 2.1.5. Cells were stained with Thy1.2-PE-Cy7, CD4-APC-H7 and V β 11-PE.

2.9 STATISTICAL ANALYSIS

P values were calculated using the Mann Whitney test performed using Prism 5.0 software (Graphpad). Survival curves were constructed using the Kaplan Meier method using Prism 5.0 software. The log rank test was used to analyze subgroups in overall survival analysis. A p value of equal to or less than 0.05 was considered significant. (In figures, * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$).

CHAPTER 3 – IN VITRO FUNCTIONAL ANALYSIS OF F5-TCR AND CD3 TRANSDUCED CD4+ T CELLS

3.1 Introduction

Our laboratory have previously explored the transduction of CD4+ T cells with the class I MHC restricted TCR, F5-TCR. The F5-TCR is specific for the influenza peptide, NP, presented by the murine class I MHC molecules, H2-D^b. CD4+ T cells that express the F5-TCR can recognize specific antigen in vitro but have a lower functional avidity than CD8+ T cells expressing the same F5-TCR (4). When stimulated with dendritic cells loaded with NP peptide, a 10 fold higher concentration of peptide was required to trigger IFN- γ secretion by F5-TCR transduced CD4+ T cells than was required by F5-TCR transduced CD8+ T cells. In addition, F5-TCR transduced CD4+ T cells did not produce IFN- γ in response to stimulation with the EL4-NP tumour cell line but NP-specific proliferation and IL-2 secretion was observed. The F5-TCR is partially CD8 dependent, and therefore in CD4+ T cells in the absence of the CD8 co-receptor, the functional avidity is lower compared to CD8+ T cells.

The functional avidity of a T cell is dependent on both the affinity of the individual TCR for the peptide-MHC complex, the density of TCR expression and the 'fitness' of the T cell. Irrespective of the affinity of the TCR for peptide-MHC, T cells will not be activated until a threshold number of TCR

have been ligated. This is not an absolute number and is dependent on other factors such as the interaction of CD8 or CD4 co-receptors. An in vitro study performed with human T cells with an estimated level of TCR expression of 30,000 TCR/cell estimated that the minimum number of TCR required for activation in the absence of co-stimulation was 8,000 and in the presence of co-stimulation was 1,000 (277). A number of studies have demonstrated that the density of TCR expression correlates with the level of T cell activation. Using a transgenic model where T cells expressed different levels of an identical TCR, it has been shown that reducing TCR number from 50,000/cell to 10,000/cell led to a 3 fold reduction in the level of calcium mobilization and also delayed T cell responses such as proliferation and IFN- γ production (278). The level of calcium mobilization seen correlated linearly with TCR expression.

Following TCR gene transfer, the expression level of the introduced TCR is typically lower than that observed in the parental T cell clone. TCR expression is dependent on how well the introduced TCR competes with the endogenous TCR for binding to CD3. If there is only one TCR present, then the majority of TCR complexes would be expected to bind efficiently to CD3 and be expressed on the cell surface. However in TCR transduced T cells, the introduced, endogenous and also any mispaired TCR heterodimers will compete for binding to the endogenous supply of CD3.

With the aim of increasing surface expression of the introduced (desired) TCR, co-transfer of additional CD3 molecules was assessed. We have

previously examined the in vitro and the in vivo functional avidity of TCR and CD3 co-transduced CD8⁺ T cells in a murine model. This study demonstrated that the amount of CD3 was rate limiting for the expression of the introduced TCR. The co-transduction of CD3 and TCR led to a 16-20 fold higher level of TCR surface expression and tetramer binding compared to TCR expression in cells that had been transduced with only the TCR genes. This observed increase in surface expression of the F5-TCR led to an improvement in functional avidity. This translated in vivo to faster eradication of tumours, secondary to more efficient trafficking to the tumour site of TCR + CD3 T cells and higher levels of T cell expansion post adoptive transfer. In addition, TCR + CD3 CD8⁺ T cells had superior recall responses following rechallenge (5).

3.2 Aims and Hypothesis

The aim of experiments reported in this chapter was to explore whether the functional avidity of a CD4⁺ T cell transduced with a class I restricted TCR could be improved by the co-transfer of additional CD3. As described above, experiments using F5-TCR expressing CD4⁺ T cells have demonstrated a reduced functional avidity compared to CD8⁺ T cells expressing the same TCR. These experiments explore initially whether additional CD3 can increase the surface expression of the F5 TCR in CD4⁺ T cells and then assess the functional profile of the transduced T cells following in vitro stimulation.

3.3 Co-transduction CD3 increases surface expression of the introduced F5-TCR in CD4+ T cells.

Co-transduction was carried out on purified populations of C57BL/6 CD4+ splenocytes. CD4+ splenocytes were purified by positive selection using anti-CD4 magnetic beads and then were polyclonally activated using anti CD3-CD28 beads. Typical purity post CD4+ selection was 95% or greater. Transduction was carried out 24 hours post activation as described in detail in Chapter 2, section 2.4.4.

CD4+ splenocytes were transduced with F5-TCR alone, co-transduced with F5-TCR and CD3-GFP or with F5-TCR and GFP control. In cells transduced with F5-TCR plus CD3-GFP, GFP expression was used to identify cells expressing endogenous CD3 (GFP negative) from cells expressing additional exogenous CD3 (GFP positive). Transduced T cells were stained with the anti-V β 11 antibody, which binds to the F5-TCR V β region.

On day 4 post transduction, viable transduced T cells were stained with antibodies to CD4 and V β 11 and analyzed by FACS to determine the purity and levels of TCR and exogenous CD3 expression. Figure 9A shows typical expression levels post transduction. CD4+ mock transduced T cells had an endogenous V β 11 expression of 5.76%. Following transduction with F5-TCR only, 87.8% of CD4+ T cells were V β 11 positive. Following transduction with F5-TCR and GFP control, 77.3% of CD4+ T cells expressed both V β 11 and

GFP. Finally, following transduction of F5-TCR and CD3-GFP, 91.7% of CD4⁺ T cells had double expression of V β 11 and CD3-GFP (Figure 9A).

The co-transduction of CD3-GFP led to an increase in surface expression of the F5-TCR. The V β 11 Mean Fluorescent Intensity (MFI) of mock transduced cells, F5-TCR CD4⁺ T cells and F5 TCR + GFP control CD4⁺ T cells were 3058, 2200 and 2083 respectively (Figure 9B). In CD4⁺ T cells co-transduced with F5-TCR and CD3-GFP the V β 11 expression MFI was 8300. Thus, co-transduction of additional CD3 molecules plus F5-TCR led to a four fold increase in V β 11 expression than that of cells transduced with F5-TCR alone or F5-TCR plus GFP-control. V β 11 MFI was equivalent in mock transduced CD4⁺ T cells and in F5-TCR only transduced CD4⁺ T cells. There was no increase in MFI seen when cells were co-transduced with F5-TCR plus GFP-control compared to cells transduced with F5-TCR only.

In addition, the transduced CD4⁺ T cells were stained with NP-pentamer to determine if increased TCR surface expression enhanced pentamer binding. Transduced cells were stained for expression of CD4 and V β 11 or for CD4 and NP-pentamer. 55.4% of CD4⁺ cells transduced with F5-TCR were able to bind NP-pentamer (Figure 10). Of the cells co-transduced with F5-TCR and CD3, 34.1% of cells were NP-Pentamer/CD3-GFP double positive. There

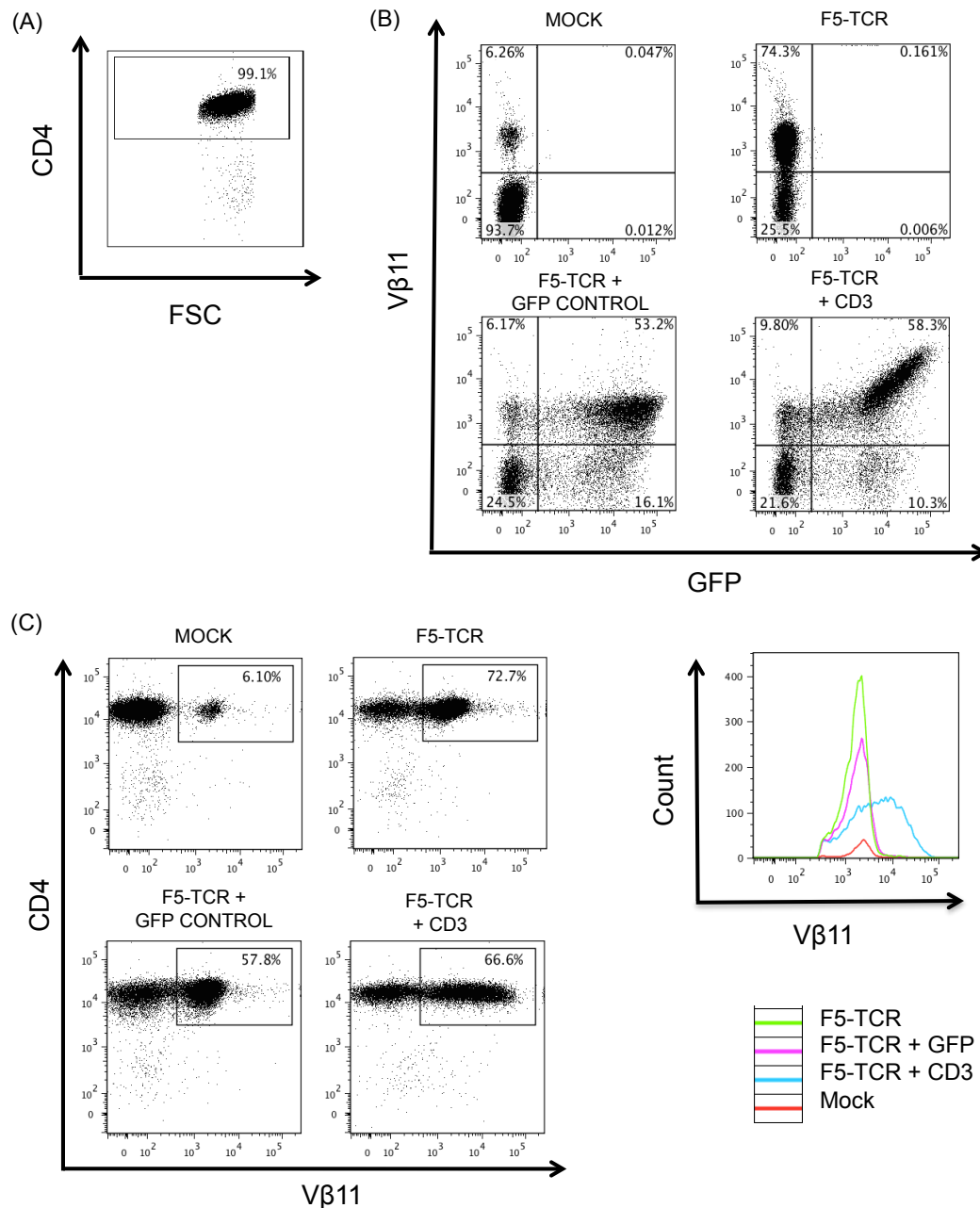


Figure 9 – Co-transfer of CD3 increases TCR expression in F5-TCR transduced CD4+ T cells. Cells were stained with antibodies to Vβ11 and CD4 prior to FACS analysis. (A) – Typical CD4+ purity of transduced T cells post transduction (Cells were gated on viable lymphocytes). (B) Transduction of CD4+ T cells with Mock, F5-TCR, F5-TCR + GFP or F5-TCR + CD3 (Cells were gated on viable lymphocytes and then on CD4+ T cells). (C) Transduction with F5-TCR and CD3 increases the MFI of Vβ11 compared to cells transduced with F5-TCR only or F5-TCR + GFP. Cells were gated on viable lymphocytes. Mock Transduced (MFI 3058), F5 TCR (MFI 2200), F5 TCR and GFP control vector (MFI 2083) and F5 TCR and CD3-GFP vector (MFI 8300) .

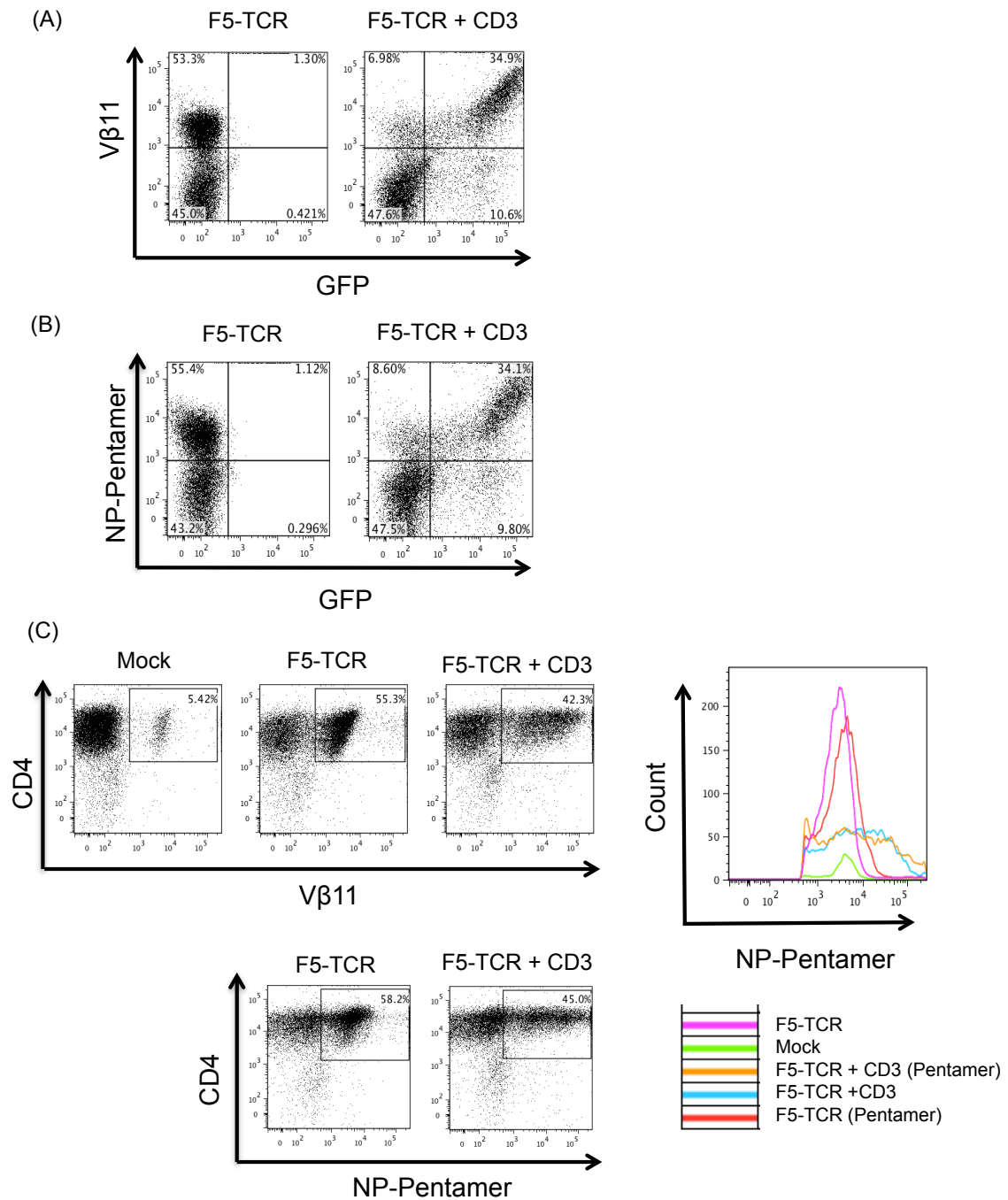


Figure 10 – Co-transfer of CD3 enhances NP-pentamer binding by F5-TCR transduced CD4+ T cells. Transduced populations were stained for expression of (A) Vβ11 or (B) binding to specific NP-pentamer. Cells had been pre-gated on CD4+ splenocytes and viable lymphocytes (C) Transduction with F5-TCR and CD3 increases the MFI of Vβ11 and of NP-Pentamer compared to cells transduced with F5-TCR only. Cells were gated on viable lymphocytes. Mock Transduced – MFI Vβ11 = 6469, F5-TCR MFI Vβ11 = 4603, F5-TCR MFI Pentamer = 5730, F5-TCR + CD3 MFI Vβ11 = 1.93×10^4 and F5-TCR + CD3 MFI Pentamer = 2.45×10^4 .

was a 4.2 fold increase in pentamer binding in the cells co-transduced with F5-TCR and CD3-GFP compared to cells transduced with F5 TCR alone (MFI NP-Pentamer F5 TCR only = 5730; MFI NP Pentamer F5 TCR-CD3 GFP = 2.45×10^4).

3.4 CD4⁺ T cells co-transduced with F5-TCR and CD3 display increased antigen-specific cytokine production and functional avidity in vitro.

Previous experiments demonstrated enhanced TCR expression following co-transfer of additional CD3. To assess whether this translated to improved function, cytokine production in response to stimulation with specific peptide was measured. IL-2 and IFN- γ production of F5-TCR only CD4⁺ T cells and F5-TCR + CD3 CD4⁺ cells were compared using ELISA and intracellular cytokine staining following stimulation with relevant or irrelevant peptide. Initial experiments were performed using peptide loaded C57BL/6 splenocytes as antigen presenting cells. C576BL/6 splenocytes express both class I and class II MHC. The CD4 co-receptor was not expected to bind the class I MHC presenting peptide to the F5-TCR so the results obtained were independent of the CD4 co-receptor.

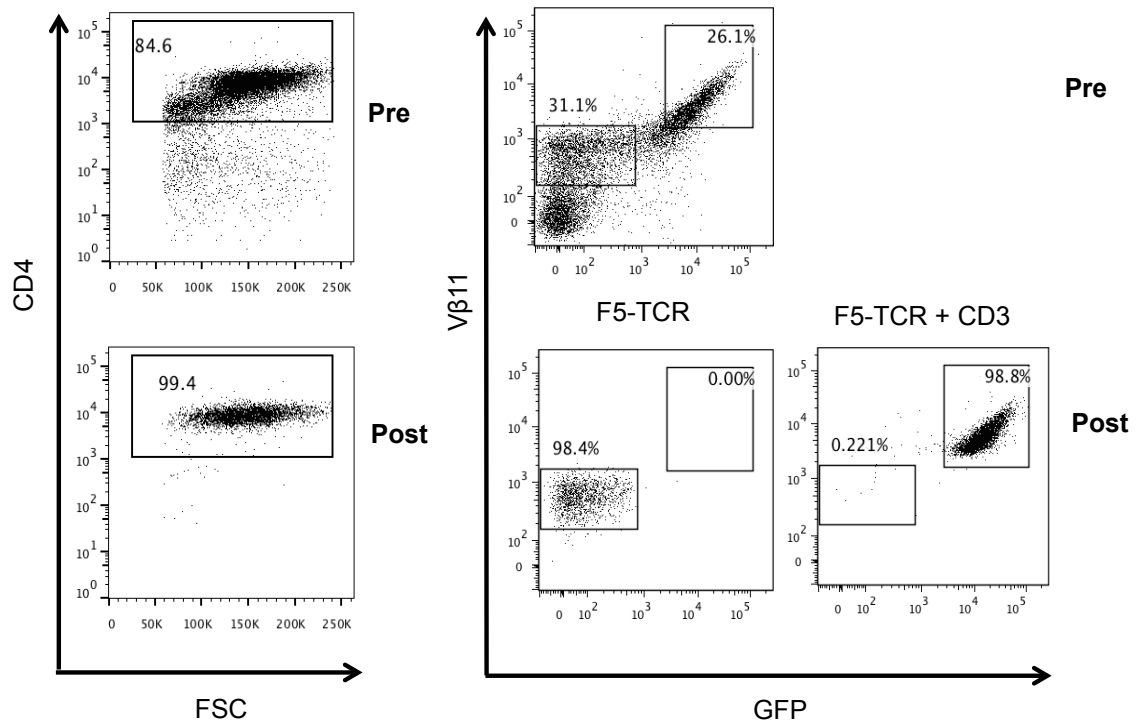
ELISA was performed on supernatants produced following overnight stimulation of TCR transduced CD4⁺ T cells with peptide loaded splenocytes in a peptide titration experiment to determine T cell avidity. Initially, the transduced CD4⁺ T cells were FACS sorted into purified populations of F5-TCR only CD4⁺ T cells and F5-TCR + CD3 CD4⁺ T cells. FACS sorting was

performed on day 5 post transduction. Figure 11A shows a representative FACS sorting profiles pre and post sort. Both F5-TCR only CD4⁺ and F5-TCR-CD3 CD4⁺ populations had >90% purity post FACS sort. The transduced cells were stimulated with peptide loaded splenocytes as described (Chapter 2, section 2.6) on the day of the FACS sort.

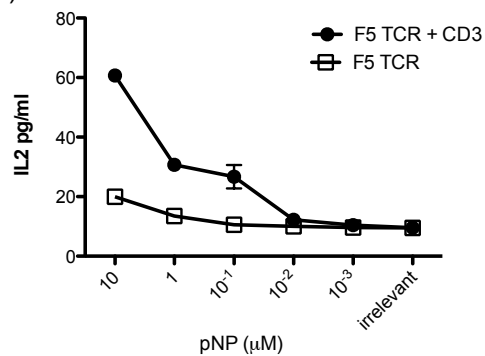
CD4⁺ T cells co-transduced with F5-TCR and CD3 produced higher levels of both IL-2 and IFN- γ following stimulation with NP peptide (Figure 11B and 11C). F5-TCR + CD3 CD4⁺ T cells were able to produce IL-2 in response to 100nm of NP peptide and above whereas TCR only CD4⁺ T cells produced lower concentrations of IL-2 in response to the highest (saturating) peptide concentration, 10 μ m. F5-TCR + CD3 CD4⁺ T cells produced IFN- γ in response to 1 μ m and above whilst no production of IFN- γ was seen following stimulation of F5-TCR only CD4⁺ T cells. The CD4⁺ T cells co-transduced with F5-TCR and CD3 were functional in vitro and produced IL-2 and IFN- γ in response to peptide stimulation. Furthermore, they produced more cytokines than F5-TCR only CD4⁺ T cells and were able to respond to lower concentrations of specific peptide. These results suggest that the higher surface level of TCR following co-transduction of CD3 improves the functional avidity of F5-TCR transduced CD4⁺ T cells.

IL-2 and IFN- γ production was also measured by intracellular cytokine staining of transduced T cells following overnight stimulation with peptide loaded splenocytes. As in the ELISA, peptide titration was performed with NP peptide to determine differences in functional avidity between F5-TCR CD4⁺

(A)



(B)



(C)

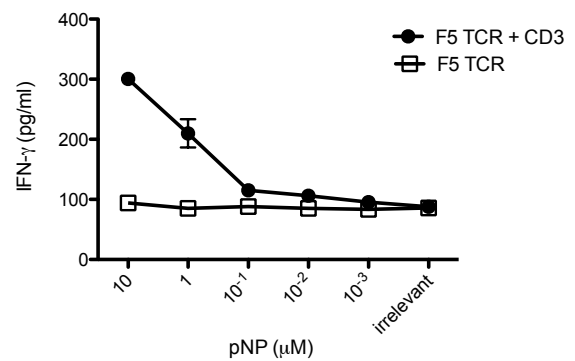


Figure 11 – F5-TCR transduced CD4⁺ T cells expressing additional CD3 have increased antigen-specific cytokine production and functional avidity in vitro. Transduced T cells were FACS sorted into purified populations of F5-TCR + CD3 CD4⁺ T cells and F5-TCR CD4⁺ T cells prior to overnight stimulation with peptide loaded C57BL/6 splenocytes. (A) – Representative plot of FACS sort profile pre and post sort following gating on viable lymphocytes. (B) IL-2 production measured by ELISA (n=3 experiments, each experiment was performed in triplicate). (C) IFN-γ production measured by ELISA (n= 2 experiments, each experiment was performed in triplicate).

T cells and F5-TCR + CD3 CD4⁺ T cells. For FACS analysis, cells were initially gated on viable CD4⁺ cells and then gated on F5-TCR only cells or F5-TCR + CD3 cells. IL-2 or IFN- γ production in each separate populations was then analyzed. Representative FACS analysis from one experiment is shown in Figure 12A and 13A. Figures 12B and 13B summarizes data from 4 individual experiments.

A higher proportion of F5 TCR + CD3 CD4⁺ T cells secreted IL-2 and IFN- γ when compared to F5-TCR only CD4⁺ T cells in response to all peptide concentrations tested. F5-TCR + CD3 CD4⁺ T cells produced IL-2 and IFN- γ following stimulation with ≥ 10 nm of NP peptide. F5-TCR only CD4⁺ T cells had demonstrable cytokine production when stimulated with saturating (10 μ m) peptide concentrations only. Thus, co-transfer of additional CD3 resulted in increased numbers of antigen responsive cells and increased functional avidity as measured by intracellular cytokine staining.

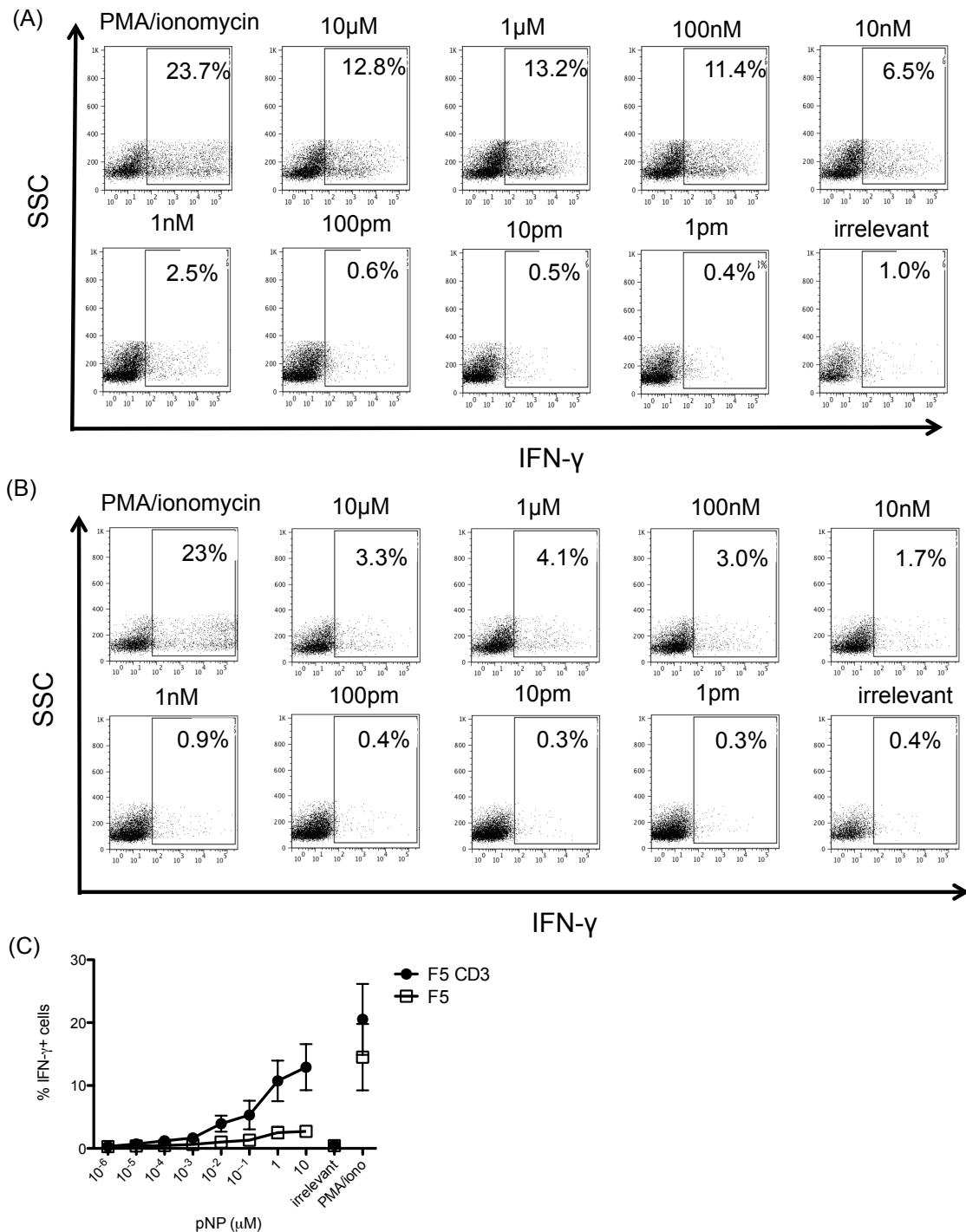


Figure 12 – Co-transfer of CD3 increases proportion of antigen specific IFN- γ secreting F5-TCR transduced CD4⁺ T cells and their functional avidity. Purified populations of F5 TCR CD4⁺ T cells or F5-TCR + CD3 CD4⁺ T cells were stimulated overnight with peptide loaded splenocytes and then fixed and permeabilized prior to staining for intracellular IFN- γ . IFN- γ production is shown following gating on viable (A) CD4⁺ F5-TCR + CD3 positive T cells (30% of CD4⁺ T cells were F5-TCR + CD3 transduced) or (B) F5-TCR CD4⁺ T cells (22% of CD4⁺ T cells were F5-TCR positive). FACS plots show results from one representative experiment. (C) Summary plot comparing % of F5-TCR or F5-TCR CD3 CD4⁺ T cells producing IFN- γ (n=4 experiments)

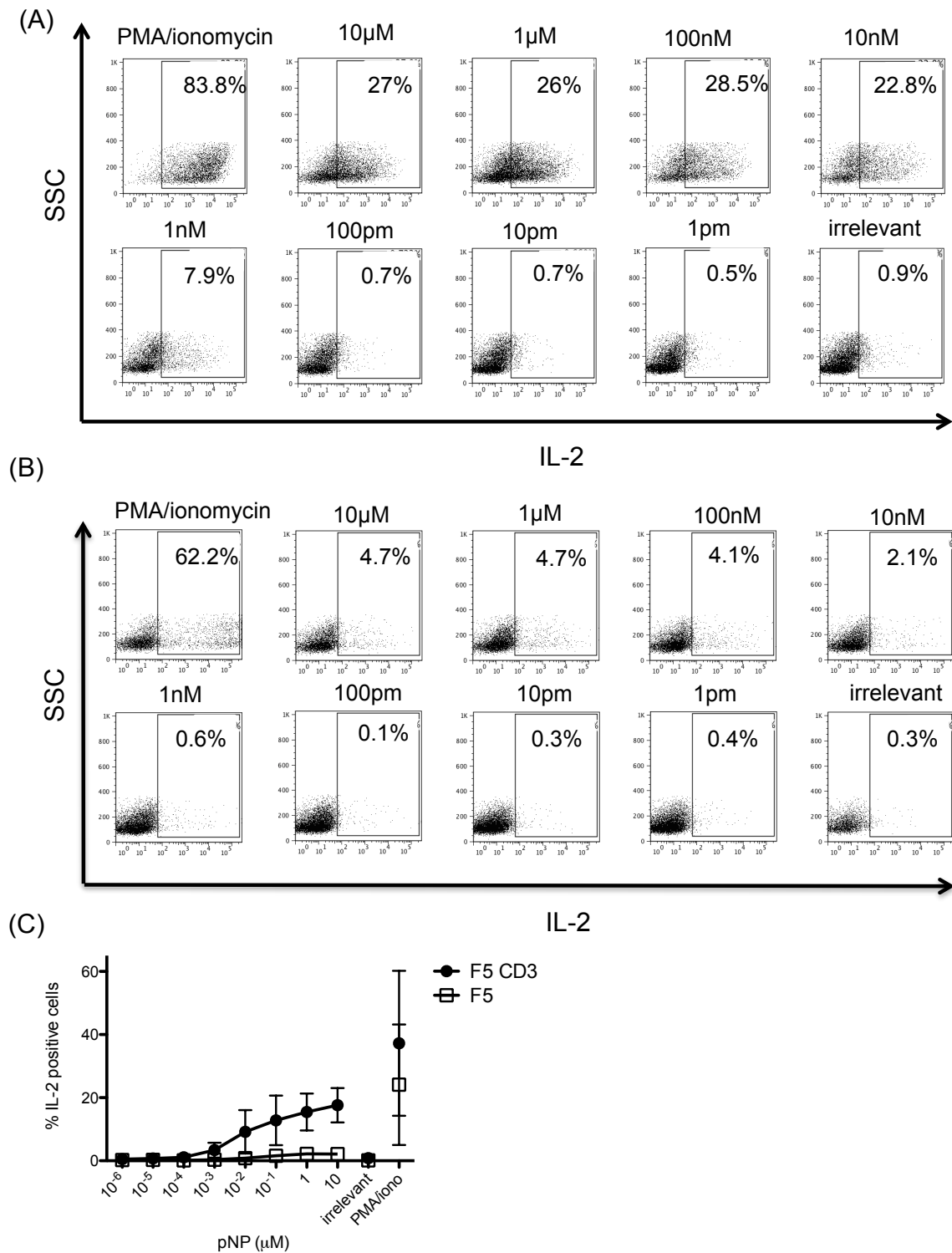


Figure 13 – Co-transfer of CD3 increases proportion of antigen specific IL-2 secreting F5-TCR transduced CD4+ T cells and their functional avidity. Purified populations of F5 TCR CD4+ T cells or F5-TCR + CD3 CD4+ T cells were stimulated overnight with peptide loaded splenocytes and then fixed and permeabilized prior to staining for intracellular IL-2. IL-2 production is shown following gating on viable (A) CD4+ F5-TCR + CD3 positive T cells (30% of CD4+ T cells were F5-TCR + CD3 transduced) or (B) F5-TCR CD4+ T cells (22% of CD4+ T cells were F5-TCR positive). FACS plots show results from one representative experiment. (C) Summary plot comparing % of F5-TCR or F5-TCR CD3 CD4+ T cells producing IL-2 (n=4 experiments)

3.5 Enhanced antigen-specific proliferation of F5-TCR transduced CD4+ T cells expressing additional CD3.

Proliferation of F5-TCR + CD3 CD4+ T cells and F5-TCR only CD4+ T cells was assessed using $\{^3\text{H}\}$ -thymidine incorporation assays as described in Chapter 2, Section 2.6. The peptide stimulation was carried out on FACS sorted CD4+ T cells as before on day 5 post transduction. Briefly, cells were stimulated for 24 hours with peptide loaded C57BL/6 splenocytes and then pulsed with $\{^3\text{H}\}$ -thymidine and incubated for a further 24 hours.

CD4+ T cells co-transduced with F5-TCR and CD3 had higher levels of antigen specific proliferation than CD4+ T cells transduced with F5-TCR only (Figure 14A, n=3 experiments). F5-TCR + CD3 CD4+ T cells proliferated in response to stimulation with 1nm of NP peptide whilst proliferation responses of F5-TCR CD4+ T cells were only seen following stimulation with 1 μm of NP peptide or above.

Proliferation was also assessed by measurement of the dilution of the cell trace dye efluor 670®. Transduced T cells were labeled with cell trace efluor 670® on day 5 post transduction and stimulated for 48 hours with peptide loaded splenocytes. Cells were then stained with surface antibodies against V β 11 and CD4 and the proliferation of transduced populations was assessed by measuring the dilution of efluor 670® dye (Figure 14B and Figure 14C). At all concentrations of NP peptide down to 1nm, F5-TCR + CD3 CD4+ T

cells had higher levels of proliferation than F5-TCR only CD4+ T cells. F5-TCR only CD4+ T cells proliferated in response to stimulation with 10nm NP peptide whilst F5-TCR + CD3 CD4+ T cells could proliferate in response to stimulation with 1nm of NP peptide.

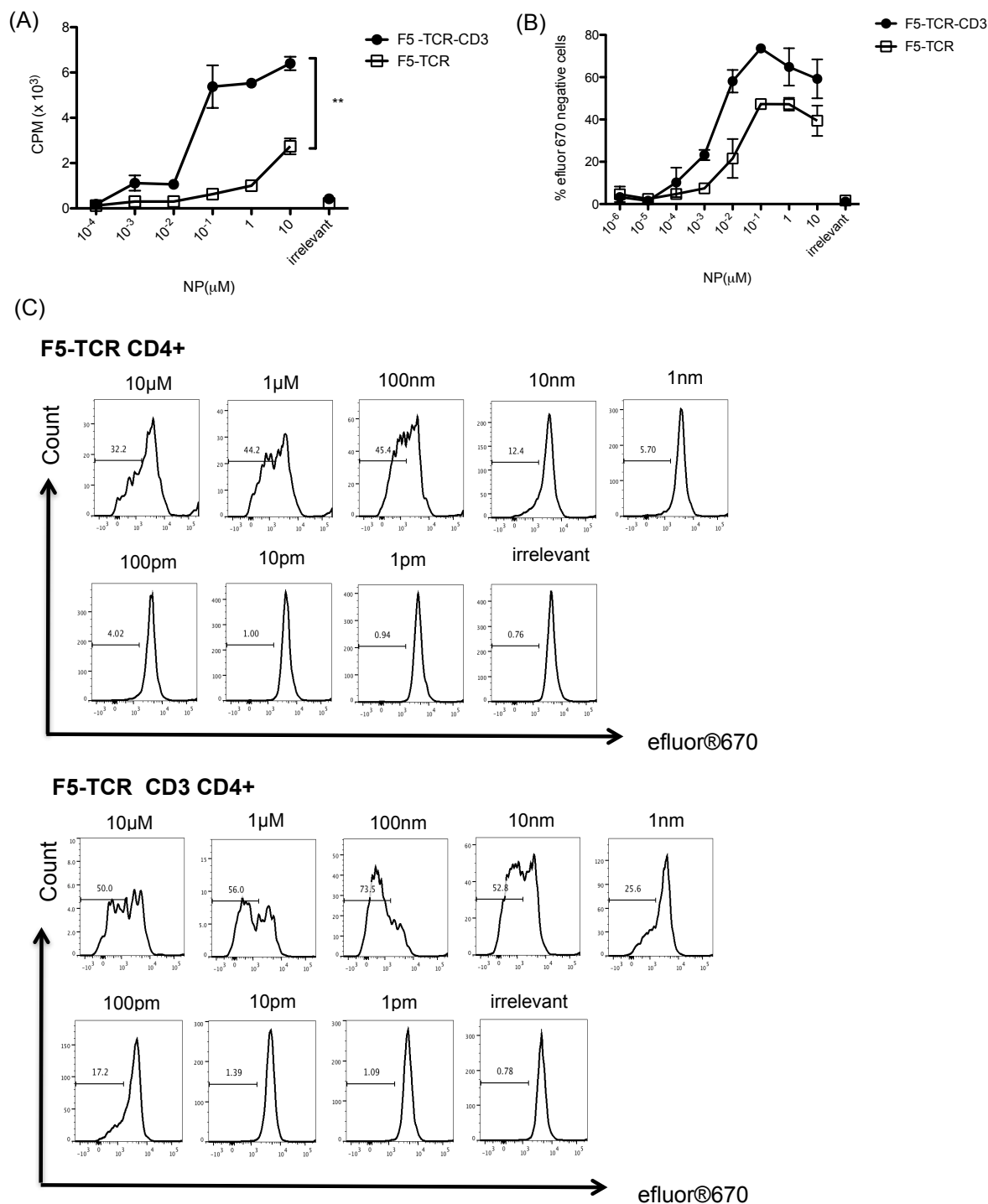


Figure 14 – F5-TCR CD3 CD4+ T cells have higher levels of proliferation in response to specific peptide than F5-TCR CD4+ T cells. (A) Proliferation measured by ^3H -thymidine incorporation following overnight stimulation with syngeneic splenocytes loaded with NP peptide. Transduced T cells were FACS sorted into purified populations of F5-TCR or F5-TCR + CD3 T cells prior to stimulation. Plots summarize results from 3 independent experiments. (B) Proliferation measured by dilution of cell trace dye efluor 670®. Proliferation of transduced CD4+ T cells was measured following 48 hour stimulation with peptide loaded syngeneic splenocytes. Transduced CD4+ T cells were pre labelled with efluor 670® cell trace dye prior to stimulation (Transduced T cells were not FACS sorted prior to stimulation). Results from 3 independent experiments are summarized in figure (B) and (C) shows representative FACS plots from one experiment. Viable cells were gated on CD4 positive cells and then on F5-TCR expressing or F5-TCR + CD3 expressing populations to assess the expression level of efluor 670.

3.6 Comparison of the in vitro function of F5-TCR CD4+, F5-TCR + CD3 CD4+ and F5-TCR + CD8+ T cells. Does additional CD3 improve the functional avidity of CD4+ T cells transduced with F5 to that of F5-TCR expressing CD8+ T cells?

Previous experiments comparing CD4+ and CD8+ T cells transduced with the identical class I restricted F5-TCR have shown that F5-TCR CD4+ T cells have lower avidity than F5-TCR CD8+ T cells (4). This is a result of the absence of binding of the CD4 co-receptor in the TCR-peptide-MHC class I interaction. The experiments described here explored whether the co-transduction of F5-TCR plus additional CD3 improved the functional avidity of CD4+ T cells to that observed for CD8+ T cells expressing the F5-TCR i.e. could increased TCR expression overcome the need for cognate co-receptor expression.

F5-TCR+ CD3 CD4+ and F5-TCR only CD8+ T cells were generated by retroviral transduction as in previous experiments. IL-2 and IFN- γ secretion by F5-TCR + CD3 CD4+ T cells was measured in response to stimulation with decreasing concentrations of specific peptide. Supernatant for ELISA was obtained following 24 hours in vitro stimulation. The transduced T cells in these experiments were not FACS sorted prior to stimulation and as a result the amount of cytokines produced was higher than that observed using FACS sorted populations. The results shown are the mean of 2 experiments (Figure 15).

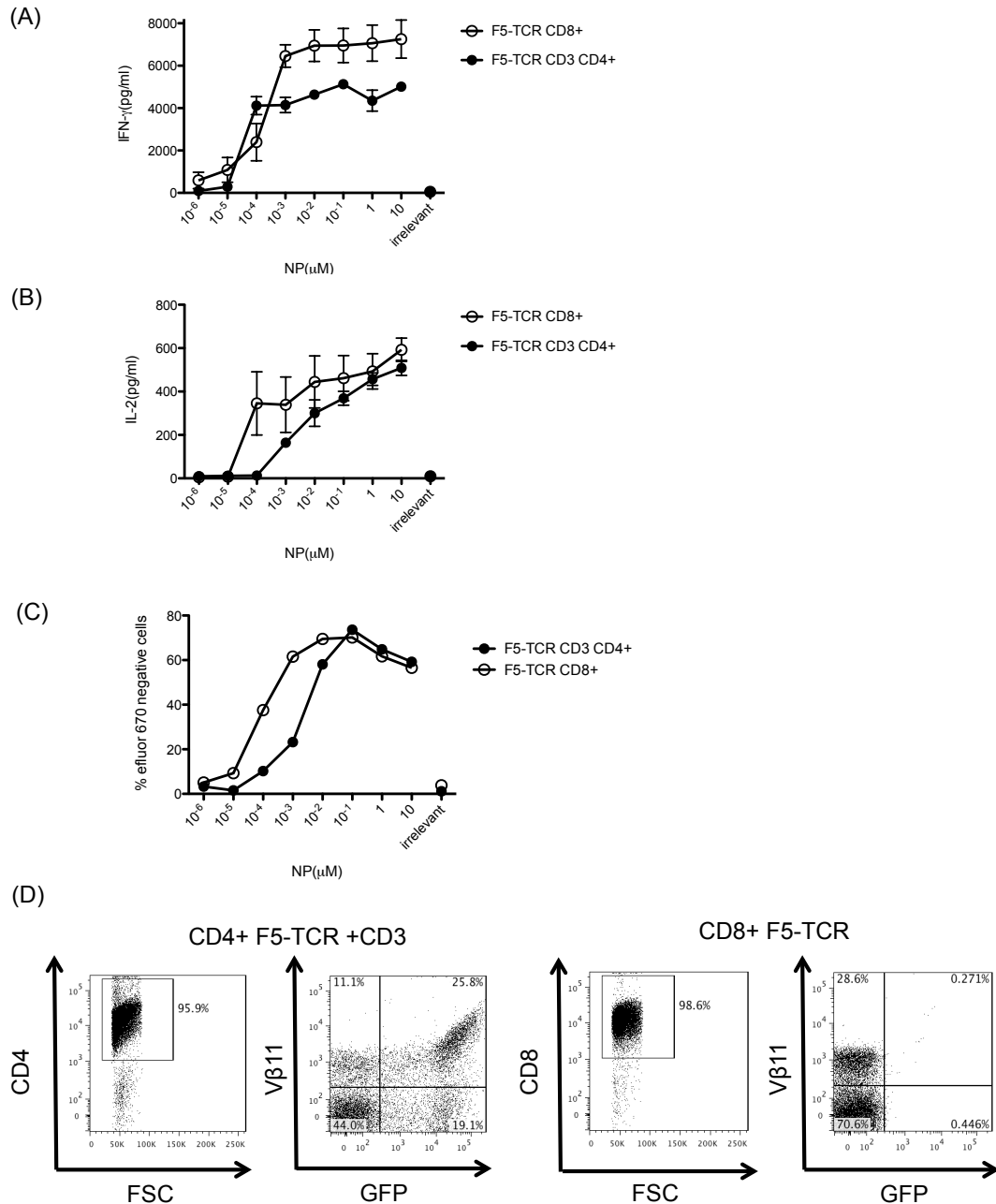


Figure 15 – In vitro antigen specific function of F5-TCR CD3 CD4+ T cells and F5-TCR CD8+ T cells. Transduced populations of F5-TCR + CD3 CD4+ T cells and F5-TCR CD8+ T cells were stimulated overnight with peptide loaded C57BL/6 splenocytes. (Transduced T cells were not FACS sorted prior to stimulation). After 24 hours of stimulation, supernatant was collected to measure IFN-γ and IL-2 production by ELISA. (A) IFN-γ production in F5-TCR CD8+ T cells and F5-TCR CD4+ T cells and ELISA measuring IL-2 production in (B) F5-TCR CD3 CD4+ T cells and F5-TCR CD8+ T cells. (C) Proliferation of F5-TCR CD3 CD4+ T cells and F5-TCR CD8+ T cells measured by dilution of effluor660 cell trace dye. (All are the mean of 2 experiments, for the ELISA the stimulations were performed in triplicate). (D) FACS plots demonstrating CD4+ or CD8+ purity of transduced cells and the % transduced cells.

For all parameters assessed (IFN- γ and IL-2 production and proliferation), CD8⁺ T cells expressing the F5-TCR had higher functional avidity than CD4⁺ T cells co-transduced with the F5-TCR and CD3. F5-TCR CD8⁺ T cells generated greater amounts of IFN- γ following stimulation with saturating concentrations of specific antigen than CD4⁺ T cells expressing the F5-TCR plus CD3. F5-TCR CD8⁺ T cells generated an IFN- γ response when stimulated with 10pm NP peptide whilst F5-TCR + CD3 CD4⁺ T cells required 100pm NP peptide to trigger an IFN- γ response. Similarly, F5-TCR CD8⁺ T cells produced more IL-2 than F5-TCR + CD3 CD4⁺ T cells. F5-TCR CD8⁺ T cells produced IL-2 in response to stimulation with 100pm NP peptide and F5-TCR + CD3 CD4⁺ T cells required 1nm of NP peptide to trigger IL-2 responses. Proliferation responses to specific antigen were seen following stimulation of F5-TCR CD8⁺ T cells with 10pm NP peptide compared to 100pm for F5-TCR + CD3 CD4⁺ T cells. These results suggest that whilst the functional avidity of F5-TCR CD4⁺ T cells is improved by the co-transduction of CD3, it did not reach equivalence to CD8⁺ T cells expressing the same TCR. The differences in functional avidity observed may related to lack of a co-receptor interaction or may be a related to inherent differences in maximal responses observable by CD4⁺ or CD8⁺ T cells. For both CD4⁺ and CD8⁺ T cells, higher concentrations of specific peptide was required to generate IL-2 than was required to generate IFN- γ or to induce proliferation.

3.7 Class I restricted CD4 T cells recognize antigen presented directly by class I expressing tumour cells.

The previous functional experiments had used splenocytes loaded with specific peptide for stimulation of the transduced T cells. Within the splenocyte suspension, there were large numbers of professional APCs such as DCs and B cells able to process and present the NP peptide to transduced T cells.

Previous work demonstrated that CD4⁺ T cells transduced with F5-TCR could produce IL-2 and proliferate in response to stimulation with EL4 tumour cells expressing NP peptide (EL4-NP cells) but they could not produce IFN- γ (4). F5-TCR expressing CD8⁺ T cells proliferated in response to stimulation with EL4-NP cells and produced both IL-2 and IFN- γ in an antigen specific manner. Experiments were designed to test whether F5-TCR + CD3 CD4⁺ T cells generate functional IFN- γ responses following stimulation with EL4-NP cells i.e. tumour cells expressing endogenously processed peptide.

F5-TCR CD4⁺ T cells and F5-TCR + CD3 CD4⁺ T cells were stimulated with either EL4-NP cells or EL4 tumour cells that do not express NP peptide and act as a negative control. Transduced T cells were stimulated for 24 hours with irradiated tumour cells and then supernatant was collected for use in ELISA as described in Chapter 2.

Both F5-TCR and F5-TCR + CD3 CD4⁺ T cells generated IL-2 and IFN- γ following stimulation with EL4-NP cells (Figure 16). Neither cell population produced cytokines in response to stimulation with EL4 cells (antigen negative). The increase in IL-2 and IFN- γ produced by F5-TCR + CD3 CD4⁺ T cells was non significant, possibly as saturating concentrations of peptide were used. These results differ from previous published data with F5-TCR CD4⁺ T cells where IFN- γ responses following stimulation with EL4-NP cells were not observed. The experiments described here utilized a second generation retroviral vector modified to optimize TCR expression. In addition, transduction efficiency of F5-TCR using the pMP71 vector was significantly higher than that using the original F5 pMX vector and this may explain the difference in functional readout that is seen.

In these experiments the class I restricted TCR transduced CD4⁺ T cells recognized antigen presented by non-professional antigen presenting cells such as tumour cells. Trans-co-stimulation provided by professional APCs in co-culture was not required for functional antigen specific responses. When control DCs (not expressing NP peptide) were added to the co-culture of transduced T cells and tumour cells the amount of cytokine produced increased. However, the presence of DCs in co-culture was not required to induce functional T cell responses to tumour cells.

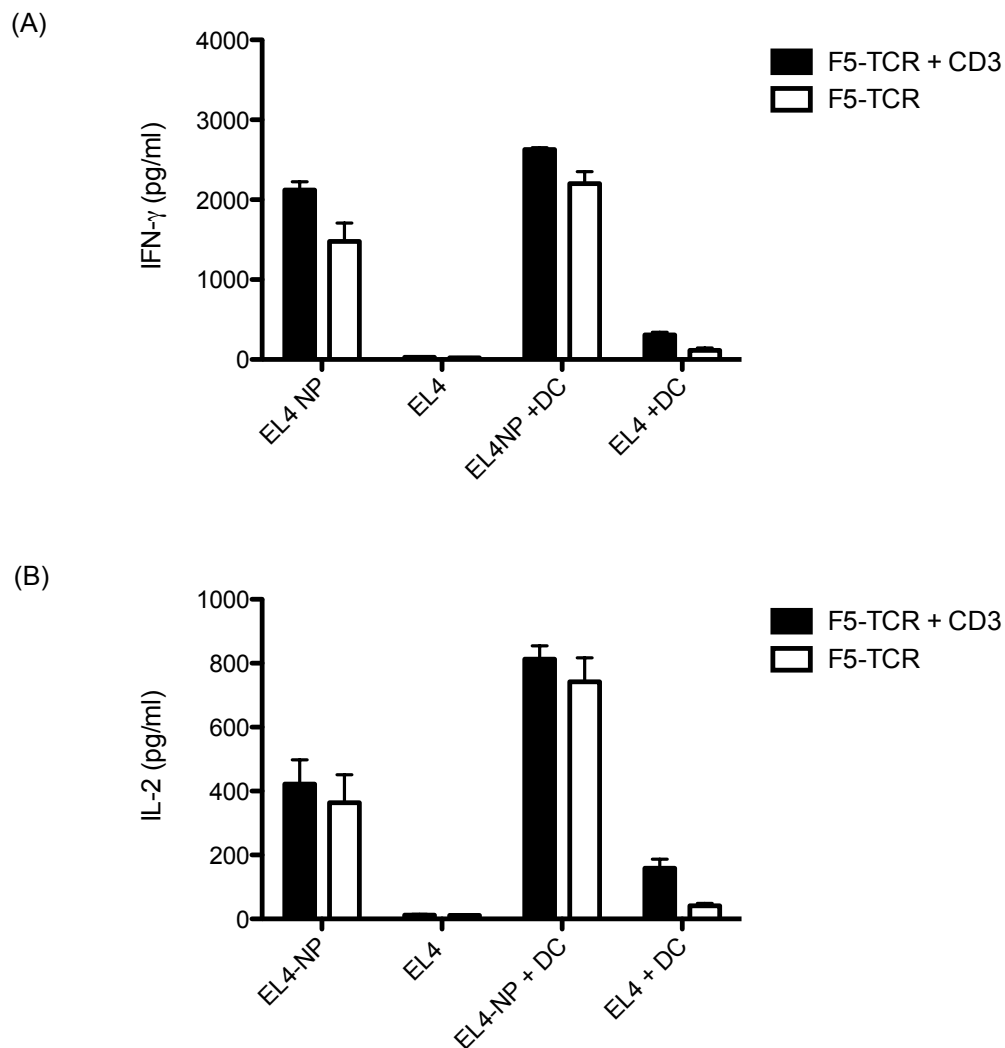


Figure 16 – F5-TCR-CD3 CD4⁺ T cells and F5-TCR CD4⁺ T cells can respond to NP peptide presented by class II negative EL4 NP tumour cells in vitro. Populations of F5-TCR + CD3 CD4⁺ T cells and F5-TCR CD4⁺ T cells were stimulated overnight with irradiated EL4 cells (NP negative and NP positive) in a 1:1 ratio of stimulators:transduced T cells +/- addition of trans-co-stimulation with dendritic cells. Transduced T cells were not FACS sorted prior to stimulation. Following 24 hours of stimulation, supernatant was collected to measure IFN- γ and IL-2 production by ELISA (A) IFN- γ production (B) IL-2 production. Results shown are the mean of 2 experiments with each stimulation being performed in triplicate).

3.8 Cytokine production profile of CD8+ and CD4+ T cells expressing the same class I restricted F5-TCR is similar but not identical.

It is not well established whether the functional profile of a CD4+ T cell is altered when it is transduced with a class I restricted TCR. Initial experiments assessing production of IFN- γ , IL-2 and proliferation demonstrated that CD4+ T cells expressing class I restricted TCR (+/- additional CD3) retain antigen specific function. However, the lack of CD4 co-receptor function may alter the activation of the CD4+ T cell and thus a qualitatively and/or quantitatively altered signal may be transduced from the TCR. Furthermore additional CD3 molecules (with a class I restricted TCR) may change the signal quality and the functional profile of the CD4+ T cell.

Experiments were performed with a luminex assay to determine the cytokine profile produced by different sorted populations of transduced T cells following stimulation with specific peptide. In these experiments, cytokine production by CD8+ T cells transduced with either F5-TCR or F5-TCR plus CD3 were compared with CD4+ T cells transduced with F5-TCR or F5-TCR plus CD3.

Supernatant was harvested following a 24 hour stimulation with syngeneic splenocytes (peptide loaded with 10 μ m of relevant or irrelevant peptide). Supernatant was assessed for presence of the following cytokines: GM-CSF, IFN- γ , IL-2, TNF- α , IL-10, IL-12, IL-17, IL-1 β , IL-4, IL-5, IL-6 and TGF- β using a luminex assay.

All four populations of transduced T cells produced measurable amounts of IFN- γ , TNF- α , and IL-2 in response to stimulation with relevant peptide. Transduced CD8⁺ T cells produced higher concentrations of all 3 cytokines, with a 5 fold increase in IFN- γ compared to transduced CD4⁺ T cells and a 2-3 fold higher concentration of TNF- α and IL-2 (Figure 17).

Antigen specific IL-4, IL-5 and GM-CSF production is shown in Figure 15. Both F5-TCR CD4⁺ T cells and F5-TCR + CD3 CD4⁺ T cells produced IL-4 and IL-5 following stimulation with relevant peptide with higher concentrations of both being produced by F5-TCR CD3 CD4⁺ T cells. F5-TCR CD8⁺ T cells or F5-TCR + CD3 CD8⁺ T cells did not produce significant amounts of IL-4 or IL-5. F5-TCR + CD3 CD4⁺ T cells, F5-TCR CD8⁺ T cells and F5-TCR + CD3 CD8⁺ T cells all produced GM-CSF in response to relevant peptide with highest concentrations being produced by F5-TCR CD8⁺ T cells (Figure 18).

There was no significant production of IL-6, IL-10, IL-12, IL-17, IL-1 β or TGF- β in response to stimulation with specific peptide by any of the 4 transduced T cell populations (data not shown).

These results suggest that class I restricted CD4⁺ T cells are poly functional and within these populations of transduced CD4⁺ T cells there are CD4⁺ T cells of a Th1 phenotype but also of a Th2 phenotype. The introduction of additional CD3 plus class I restricted TCR does not appear to change the functional profile of either CD4⁺ or CD8⁺ T cells. The cytokine profile produced by the transduced CD4⁺ T cells does not demonstrate the presence

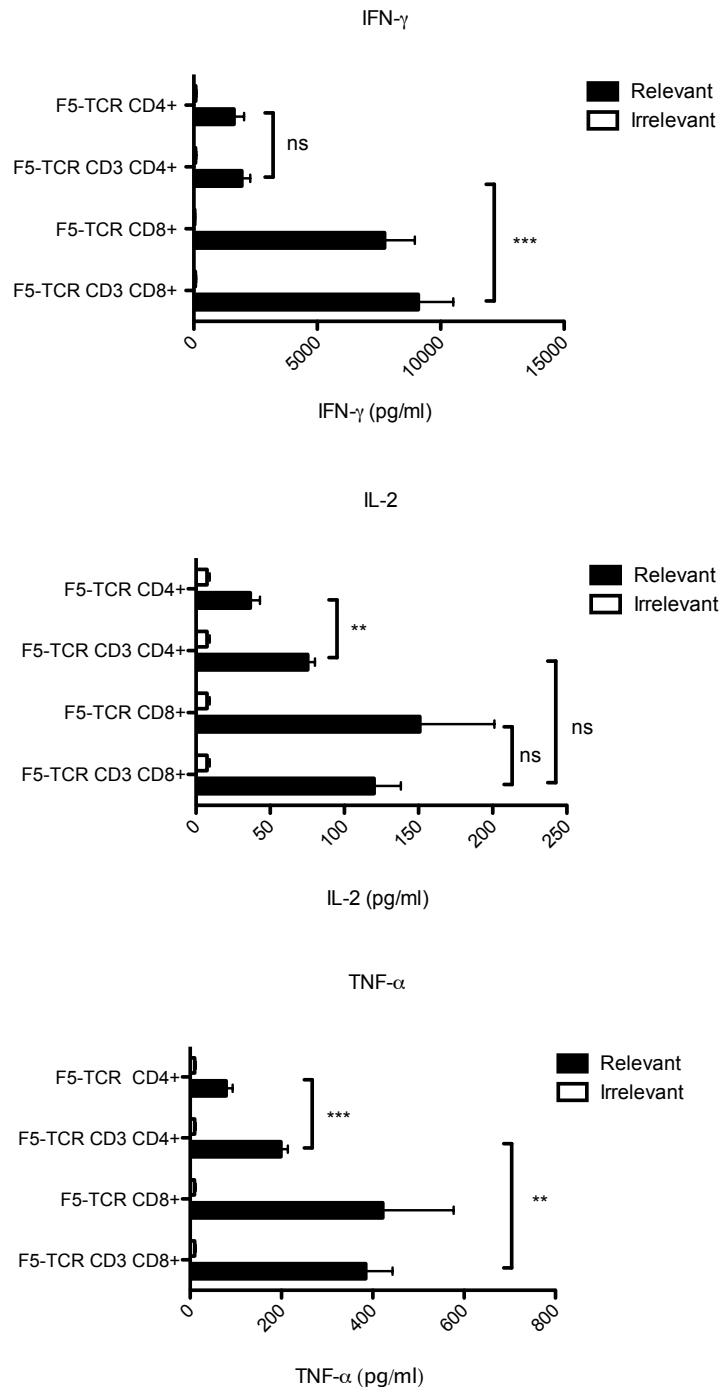


Figure 17 – CD4⁺ T cells expressing class I restricted TCR are polyfunctional, displaying a predominantly Th1 phenotype. Luminex assay was carried out using supernatants obtained following overnight culture of TCR transduced T cells in a 1:1 mix with syngeneic splenocytes peptide loaded with 10 μ m relevant or irrelevant peptide (100,000 TCR positive T cells: 100,000 peptide loaded splenocytes per condition). Transduced T cells were not FACS sorted prior to stimulation. IFN- γ , TNF- α and IL-2 production by F5-TCR + CD3 CD8⁺, F5-TCR CD8⁺, F5-TCR + CD3 CD4⁺ and F5-TCR CD4⁺ T cells following overnight stimulation is shown. (Results shown are the result of 3 independent experiments with each sample per experiment being performed in triplicate.)

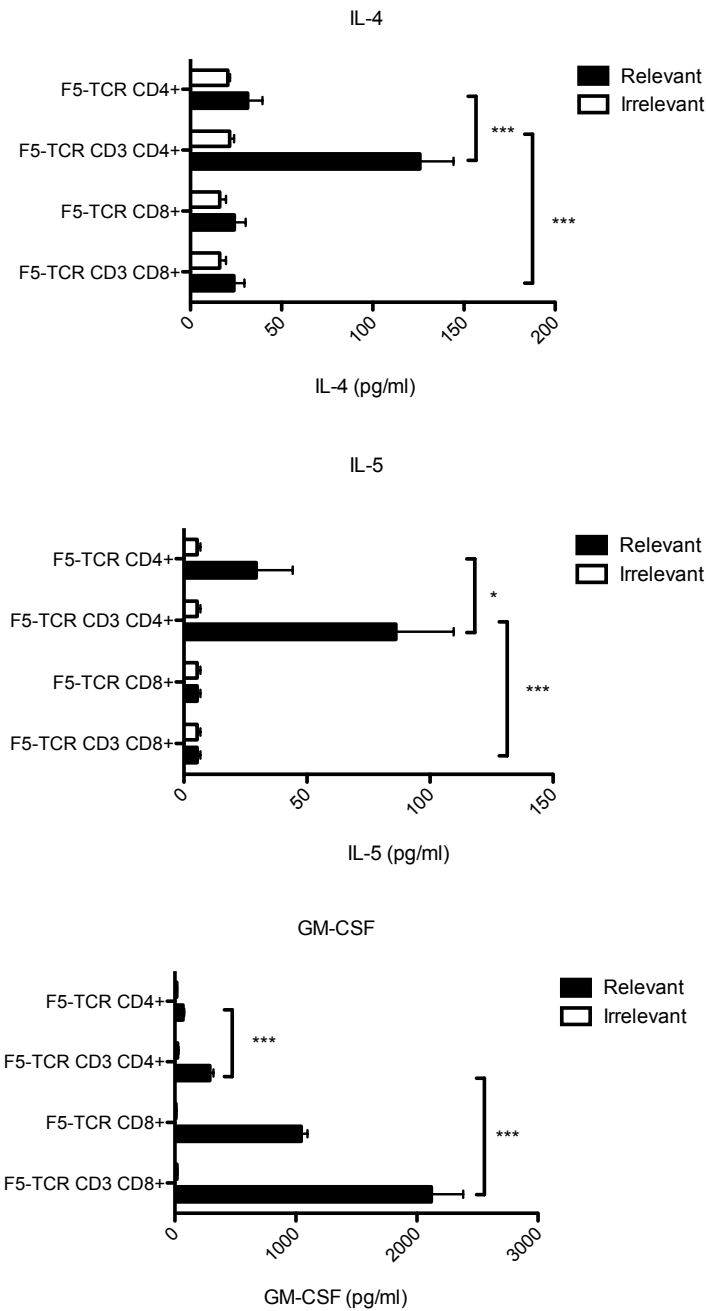


Figure 18 – CD4+ T cells expressing a class I restricted TCR also have features of Th2 CD4+ T cells. Luminex assay was carried out using supernatant's obtained following overnight culture of TCR transduced T cells in a 1:1 mix with syngeneic splenocytes peptide loaded with 10 μ m relevant or irrelevant peptide (100,000 TCR positive T cells: 100,000 peptide loaded splenocytes). T cells were not FACS sorted prior to stimulation. IL-4, IL-5 and GM-CSF and production by F5-TCR + CD3 CD8+, F5-TCR CD8+, F5-TCR + CD3 CD4+ and F5-TCR CD4+ T cells following overnight stimulation are shown. (Results shown are the result of 3 independent experiments with each sample per experiment being performed in triplicate.)

of a significant population of Th17 CD4⁺ T cells or of T regulatory CD4⁺ T cells.

3.9 Discussion

One strategy to try to improve the anti-tumour function of TCR transduced T cells is to improve the functional avidity of the tumour specific T cell for its tumour antigen. T cells with higher functional avidity can be generated by increasing the affinity of the individual TCR or by increasing the surface expression of the introduced TCR. This is particularly important when transducing a CD4⁺ T cell with a class I restricted TCR. Introduction of a class I restricted TCR into CD4⁺ T cells does not always generate fully polyfunctional CD4⁺ T cells. This may manifest as a reduced avidity compared to that seen in the parental CD8⁺ T cell or may result in a CD4⁺ T cell that can't respond to specific antigen or does not display the full range of antigen specific responses as that of a CD8⁺ T cell expressing the same TCR. CD4⁺ T cells transduced with a class I TCR have been shown to be of lower functional avidity than CD8⁺ T cells expressing the same TCR, which will ultimately affect their efficacy when translating this strategy into clinical practice. Providing a surplus of CD3 molecules by co-transferring additional CD3 plus the introduced TCR reduces potential competition for binding with CD3 that may exist between introduced and endogenous TCR and thus should enhance the surface expression of the introduced class I restricted TCR. The amount of TCR expressed on the T cell surface is one of the key determinants of functional avidity of the T cell and thus this should lead to

enhanced avidity even in the absence of a functional CD8 co-receptor interaction with MHC-I.

These experiments have shown that the co-transduction of F5-TCR and CD3 enhances the surface expression of the introduced TCR as demonstrated by staining for the V β 11 region of the F5 TCR. In addition this increase in F5-TCR surface expression leads to increased binding to specific NP pentamer. No increase in F5-TCR expression was seen following co-transduction of F5-TCR and GFP control, demonstrating that this increase in expression was as a direct result of provision of additional CD3 molecules in parallel with the introduced F5-TCR.

Following in vitro stimulation, CD4⁺ T cells expressing F5-TCR + CD3 had superior function than F5-TCR CD4⁺ T cells. F5-TCR CD4⁺ T cells could only produce IFN- γ and IL-2 and undergo proliferation following stimulation with the highest saturating concentrations of cognate antigen whilst CD4⁺ T cells co-transduced with F5-TCR and CD3 could respond to at least a 2 log fold lower concentration of specific peptide. At all concentrations of peptide stimulation, the antigen responses of F5-TCR + CD3 CD4⁺ T cells were of greater magnitude than that of F5-TCR CD4⁺ T cells. However, when compared to CD8⁺ T cells, F5-TCR + CD3 CD4⁺ T cells were still of lower avidity than that of F5-TCR CD8⁺ T cells and responses were of smaller magnitude. So it would appear that whilst improving the avidity of a class I restricted CD4⁺ T cell, the provision of additional CD3 molecules did not improve the avidity to the level of the CD8⁺ T cell expressing the F5 TCR.

The cytokine profile of class I restricted CD4⁺ T cells was not altered by the co-transfer of additional CD3. Both populations of CD4⁺ T cells transduced with either F5-TCR only or F5-TCR + CD3 demonstrated production of identical cytokine profiles, with production of both Th1 type and Th2 type cytokines. Similarly, CD8⁺ T cells produced the same cytokines whether expressing F5-TCR only or F5-TCR plus CD3. Whilst increasing the functional avidity of the CD4⁺ T cell, the co-transduction of CD3 did not alter its in vitro function. Class I restricted CD4⁺ T cells produced both Th1 type cytokines and Th2 type cytokines. There was no production of IL-17 or TGF- β or IL-10 seen by the CD4⁺ T cells which would suggest that there were not significant numbers of Th17 cells or CD4⁺ T regulatory T cells generated following TCR transduction with this TCR or with co-transfer of additional CD3.

Both F5-TCR CD4⁺ T cells or F5-TCR + CD3 CD4⁺ T cells responded in vitro to antigen presented directly by class I MHC by tumour cells, suggesting that following in vivo transfer, CD4⁺ T cells expressing class I restricted TCRs may target tumour cells directly in addition to responding to antigen cross presented by professional antigen presenting cells.

Thus the co-transfer of additional CD3 can improved the functional avidity of CD4⁺ T cells transduced with a class I restricted TCR. This produces CD4⁺ T cells that demonstrate antigen specific cytokine production and proliferation in vitro even in the absence of binding of CD8 to class I MHC. However the functional avidity of the F5-TCR-CD3 CD4⁺ T cells did not increase to the

level of a CD8⁺ T cell transduced with F5-TCR without additional CD3. The CD4⁺ T cells generated in this manner thus appear to be of intermediate avidity and it may be that class I restricted CD4⁺ T cells cannot achieve the same avidity as that of the parent CD8⁺ T cell in the absence of the CD8 co-receptor.

The function of a class I restricted CD4⁺ T cell can also be improved by the co-transduction of additional CD8 molecules which can result in enhanced functional avidity and recovery of antigen specificity in class I restricted CD4⁺ T cells. There has been no head to head comparison comparing the effects of co-transduction of CD8 versus the co-transduction of CD3 into class I restricted CD4⁺ T cells.

Xue et al transduced human CD4⁺ T cells with HLA-A2 restricted TCRs that were specific for human EBV and CMV epitopes. Transduction of CD4⁺ T cells with the class I restricted TCRs generated CD4⁺ T cells with a 10 fold lower functional avidity than CD8⁺ T cells expressing the identical TCR. This impaired avidity was entirely due to lack of CD8 co-receptor as the avidity of the CD4⁺ T cell could be corrected to that of the CD8⁺ T cell by co-transfer of TCR and CD8 into CD4⁺ T cells. These high avidity CD4⁺ T cells maintained the identical cytokine production profile to that of transduced CD4⁺ T cells that lacked additional CD8 molecules. In a xenogenic NOD/SCID mouse model, human CD4⁺ T cells that co-expressed HLA-A2 virus specific T cells and additional CD8 could efficiently eradicate tumours expressing specific EBV or CMV antigens. CD8⁺ T cells were however protective at a lower cell

dose than that of CD4⁺ T cells co-transduced with TCR and CD8, although both displayed the same functional avidity in vivo. No off or on target toxicity was demonstrated following adoptive transfer of CD4⁺ T cells co-transduced with CMV or EBV TCR plus additional CD8 co-receptor (279). Thus the transduction of additional CD8 molecules into the CD4⁺ T cells can increase the functional avidity of the CD4 T cell irrespective of the original affinity of the transduced TCR. This may represent a safer method of increasing the functional avidity of a class I restricted CD4⁺ T cell than the provision of additional CD3. When additional CD8 co-receptor is transduced there is no risk of upregulation of the expression of the endogenous TCR. In addition, the functional avidity of the CD4⁺ T cell would not be increased above that which would be expected by a CD8⁺ T cell expressing the identical TCR. The density of TCR expression and the provision of the CD8 co-receptor interaction are both key determinants of the functional avidity of a class I restricted CD4⁺ T cell. Whilst both methodologies lead to enhanced antigen specific function, there is the risk that by enhancing the functional avidity to higher levels by co-transducing additional CD3, there is potential that cross reactivity to self antigen may be revealed.

Class I restricted TCR have been described as CD8 dependent or CD8 independent. The absence of the CD8 interaction with pMHC may inhibit T cell activation to differing extents for different agonist ligands (280). The effect of the CD8 co-receptor is likely to be more apparent for weak rather than for strong pMHC-TCR interactions (281). Holler et al have suggested that CD8 dependence is controlled by the affinity of the TCR for pMHC. In T

cell hybridomas that lacked CD8, cells expressing TCR that had an affinity for pMHC above a threshold level were able to make robust antigen specific responses whilst those with TCR below a certain threshold were unable to respond (121). CD8 is shown to affect both the on rate (282) (123) and the off rate (124) of the TCR-pMHC interaction. It has been suggested that for high affinity TCR that the signaling function of CD8 is most important whilst for TCR with lower affinity both the signaling and the stabilization of the TCR-pMHC provided by CD8 are important (283).

The efficient regulation of CD8 T cell activity may be provided via modification of the CD8 co-receptor function. Variations in the glycosylation state and the level of expression of CD8 following antigen binding in vivo can affect the binding of TCR to pMHC (284) (285). Therefore inhibition of the extracellular binding of CD8 to pMHCI may regulate TCR antigen recognition independent from the signaling properties of the CD8 coreceptor. The recognition of all syngeneic pMHC I epitopes can be improved by the additional activity of CD8.

Affinity maturation has been used to try to increase the functional avidity of T cells. An affinity matured TCR which had a 1000 fold increased TCR affinity was introduced into CD4+T cells where it retained antigen specificity but the identical TCR expressed within CD8+ T cells developed non specific cross reactivity due to additional interaction of the CD8 co-receptor. Blockade of the CD8-MHC-I interaction with an anti-CD8 antibody prevented this cross reactivity. TCR with only modest increases in affinity retained their antigen

specificity whether expressed within CD4 or CD8 T cells. (286). It is likely that CD8 plays a vital role in modulating the functional avidity of T cells and thus the presence or absence of CD8 in class I restricted TCR expressing CD4+ T cells may have a profound affect on function. Increasing the surface expression of the class I restricted TCR by additional CD3 doesn't appear to provide the same increase of avidity as that of provision of a functional co-receptor interaction. Future experiments could be carried out to directly compare CD4+ T cells transduced with class I restricted TCR plus CD3 with CD4+ T cells transduced with the identical TCR plus CD8. It would also be interesting to explore whether the provision of additional CD3 can improve the function of a class I restricted TCR that does require the presence of a CD8 co-receptor in order to produce functional antigen specific responses. This could be used to explore responses in CD8 T cell where the CD8 co-receptor interaction was blocked and also in CD4+ T cells which lacked the CD8 co-receptor.

CHAPTER 4 – IN VIVO FUNCTIONAL ANALYSIS OF TCR TRANSDUCED CD4+ T CELLS MODIFIED TO EXPRESS ADDITIONAL CD3 MOLECULES

4.1 Introduction

The data described in Chapter 3 demonstrated that CD4+ T cells co-transduced with TCR and CD3 had enhanced functional avidity in vitro. Tumour challenge experiments were designed to assess whether class I TCR expressing CD4+ T cells could provide tumour protection in vivo following adoptive transfer and whether transduction of additional CD3 enhanced tumour protection. CD4+ T cells play a central role in tumour protection and the importance of transferring antigen specific CD4+ T cells together with antigen specific CD8+ T cells to maximize in vivo help has been demonstrated in murine models and human clinical studies. There are also a number of reports of tumour specific CD4+ T cells having the ability to eradicate tumours directly even in the absence of antigen specific CD8+ T cells (255) (262) (266).

The experiments described here explore the effects of tumour specific CD4+ T cells in isolation without tumour specific CD8+ T cells. Previous tumour challenge experiments utilizing F5-TCR transduced CD8+ T cells have demonstrated that F5-TCR CD8+ T cells can efficiently reject NP expressing tumours in vivo in the absence of tumour specific CD4+ T cells (4). In these current experiments, recipient mice received sub lethal irradiation, which depleted endogenous CD8+ T cells thus limiting their role in tumour

regression. The tumourcidal effects observed can be attributed to a direct effect of adoptively transferred tumour specific CD4⁺ T cells (including the provision of help to innate immune cells within the recipient mice).

This murine tumour model used syngeneic Thy 1.2⁺ C57BL/6 mice as recipients and C57BL/6 Thy 1.1⁺ mice as donors unless otherwise stated. In initial experiments, recipient mice received sub lethal irradiation (5.5 Gy) resulting in lymphodepletion of the host haematopoiesis and promoting homeostatic proliferation of adoptively transferred T cells. Recipient mice were irradiated on Day 0, and then 4 hours post irradiation received a subcutaneous tumour challenge with EL4-NP tumour cells. On day 1 (24 hours post irradiation), mice received intravenous injection of TCR transduced CD4⁺ T cells. The EL4 tumour cell line is a lymphoma derived from a C57BL/6 background. It only expresses class I MHC (H2-D^b) and does not express class II MHC molecules. The EL4-NP cell line has been stably transfected with the NP-peptide, which is recognized by the F5-TCR. For the initial tumour challenge experiments, EL4-NP luciferase⁺ cells were used. Tumour growth was monitored by bioluminescence imaging and caliper measurement of the tumour area. Figure 19 shows a schematic representation of the experimental set up for tumour challenge experiments.

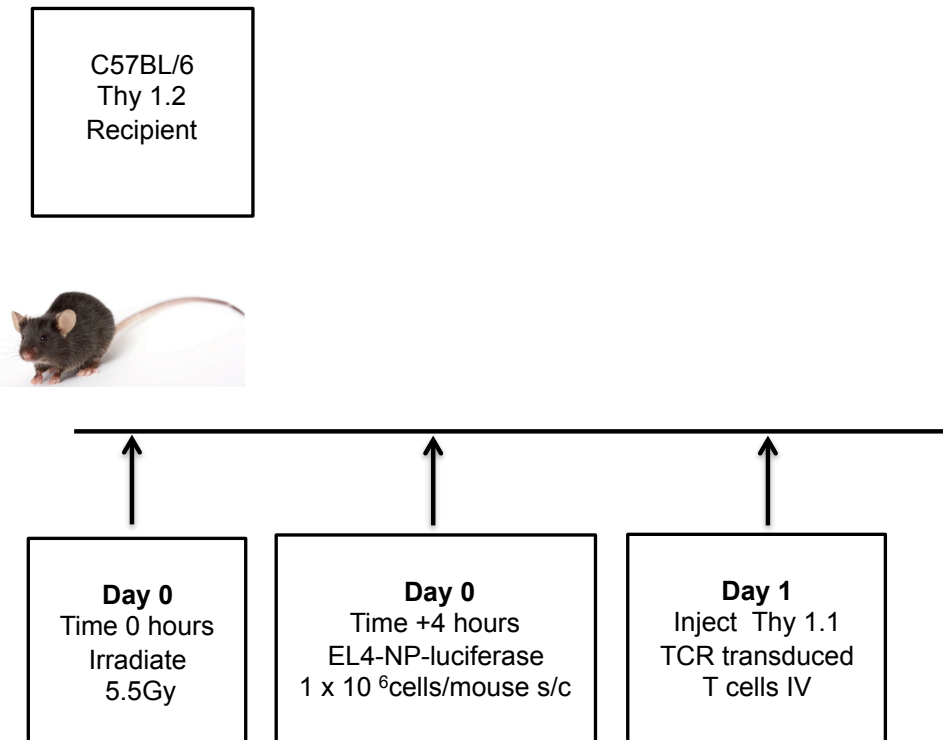


Figure 19 – Schematic representation of experimental set up for in vivo tumour protection experiments. Specific doses of radiation, tumour cells and T cells are indicated in the relevant text.

4.2 Aims and Hypothesis

It was hypothesized that the adoptive transfer of F5-TCR + CD3 CD4⁺ T cells would confer superior tumour protection than F5-TCR CD4⁺ T cells due to enhanced T cell expansion following recognition of NP expressing EL4 tumour cells. In addition F5-TCR + CD3 CD4⁺ T cells were predicted to traffic more efficiently to the tumour site than F5-TCR CD4⁺ T cells. It was expected that the adoptive transfer of F5-TCR + CD3 CD4⁺ T cells would not lead to harmful off target effects post adoptive transfer. As in vitro experiments confirmed increased antigen specific functional avidity, further in vivo experiments were carried out to determine whether CD4⁺ T cells expressing a class I restricted TCR responded to antigen presented by non professional APCs such as tumour cells.

4.3 Adoptive Transfer of F5-TCR CD3 CD4⁺ T cells confers enhanced tumour protection in vivo.

Initial tumour challenge experiments were performed to compare tumour protection provided following the adoptive transfer of F5-TCR CD4⁺ T cells and F5-TCR + CD3 CD4⁺ T cells. On day 0, recipient mice were irradiated with 5.5 Gy and 4 hours later were injected subcutaneously with 1×10^6 EL4-NP-luciferase⁺ cells. On day 1, mice received adoptive transfer of 1×10^6 TCR positive or mock transduced CD4⁺ T cells. Cells were FACS sorted prior to adoptive transfer and Figure 3A shows purity of F5-TCR CD4⁺ and F5-TCR + CD3 CD4⁺ T cells prior to injection. There were 3 different

treatment groups: Mock Transduced CD4⁺ T cells, F5-TCR CD4⁺ T cells and F5-TCR + CD3 CD4⁺ T cells, each with eight mice per group.

Mice were monitored for tumour growth, weight and clinical severity score on alternate days. Tumour size was measured with calipers and by bioluminescence imaging. As per Home Office license, mice with specified high clinical severity score, progressive tumour growth exceeding 15mm in any diameter or with >20% baseline weight loss were culled. Figure 20 demonstrates the mean tumour growth measured by tumour area and bioluminescence imaging.

All mice had measurable tumour burden by day 6 following tumour challenge. In mice receiving mock transduced CD4⁺ T cells, progressive tumour growth was observed in all mice. Tumour growth progressed at a similar rate in mice receiving F5-TCR CD4⁺ T cells. In both groups of mice, progressive tumour growth resulted in sacrifice between 13 and 18 days post T cell transfer. Measurements of tumour growth (by calipers and bioluminescence imaging) demonstrated protection in mice that receiving F5-TCR + CD3 CD4⁺ T cells. In this group, tumours grew steadily to day 12 post challenge and then tumour growth plateaued. However, complete tumour regression was only observed in 1/8 mice in this group. None of the mice within the F5-TCR + CD3 CD4⁺ group died of progressive tumour growth. All mice in this group had to be killed before the end of the experiment due to weight loss of >20% from baseline or an increased clinical severity score. This toxicity arose between days 12 and day 20 post tumour challenge. The weight loss and high severity

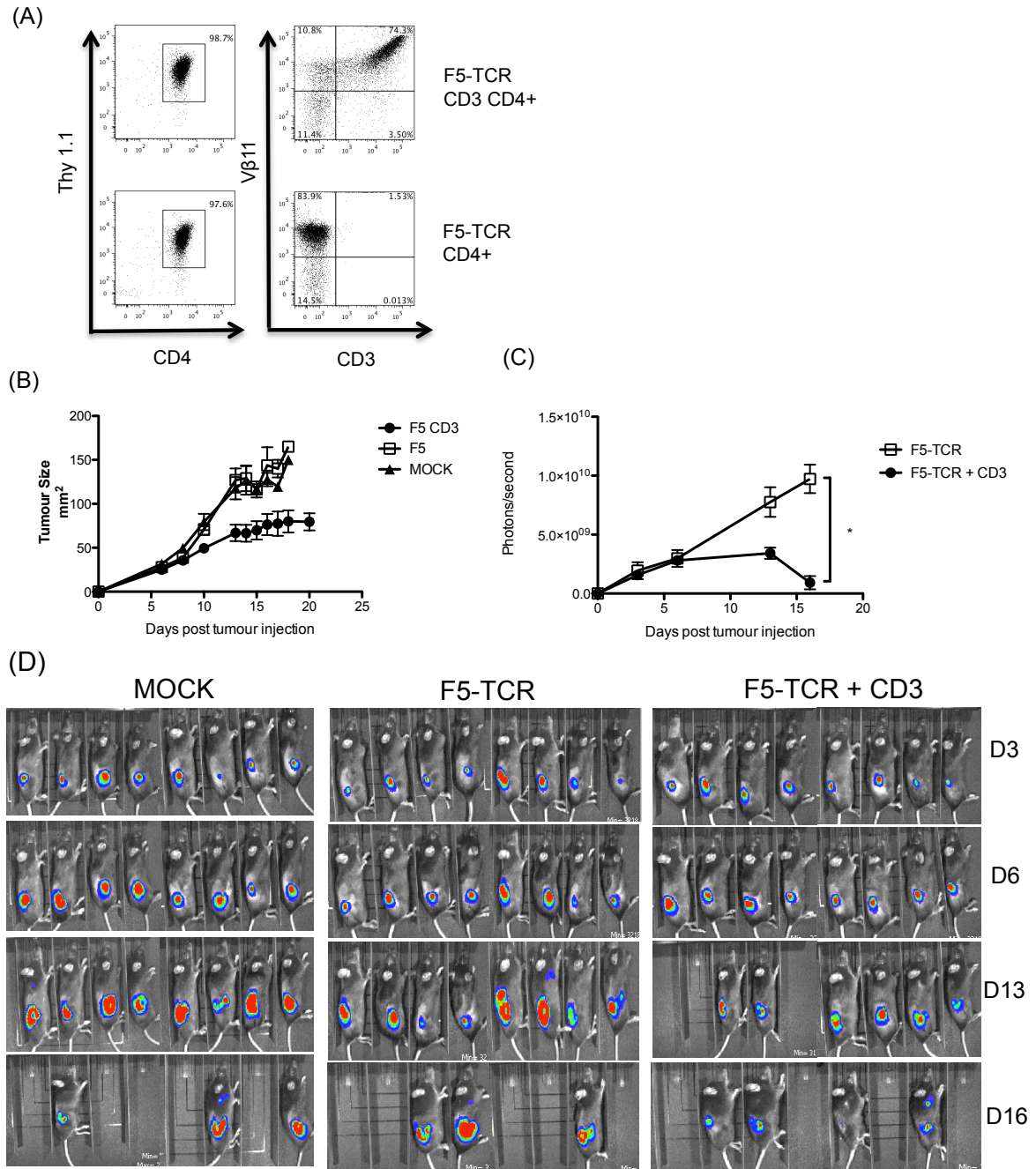


Figure 20 –Enhanced tumour protection following adoptive transfer of F5-TCR + CD3 CD4+ T cells. C57BL/6 Thy 1.2 donor mice received sub lethal irradiation with 5.5 Gy on day 0 and 4 hours later received 1×10^6 luciferase expressing EL4-NP tumour cells subcutaneously. On Day 1, mice received 1×10^6 F5-TCR, F5-TCR + CD3 or mock transduced CD4+ T cells intravenously. Results shown are from 1 experiment with 8 mice/group. (A) FACS plots of FACS sorted F5-TCR + CD3 CD4+ and F5-TCR only CD4+ T cells prior to adoptive transfer. (B) Mean tumour area measured by calipers. (C) Mean bioluminescent signal in photons/second recorded at site of tumour growth. (D) Bioluminescent signal at site of tumour growth between day 3-16 days post tumour challenges. For measurement of bioluminescent signal, mice were always arranged in identical order on each day of measurement. All mice within mock and F5-TCR group died of progressive tumour growth. Within the F5-TCR + CD3 group, no mice died of progressive tumour growth but all mice died of toxicity related to TCR transduced T cells.

score could not be attributed to tumour growth as all of the mice had low tumour burden (as determined by bioluminescence) and/or had started to regress. None of the mice that received mock transduced CD4⁺ T cells or F5-TCR CD4⁺ T cells developed cachexia or severity scores above that expected from sub lethal irradiation or from progressive tumour growth.

The finding of increased toxicity within the recipients of F5-TCR + CD3 CD4⁺ T cells was confirmed in a second independent experiment. The experimental set up was similar to the previous experiment although luciferase negative tumour cells were used for challenge and the number of mice per group was 5. In this experiment, all mice receiving mock transduced CD4⁺ T cells had progressive tumour growth between day 11 and 12 post tumour challenge. All mice that received F5-TCR CD4⁺ T cells had progressive tumour growth that occurred between day 11 and day 19 post tumour challenge. All of the mice within the F5-TCR + CD3 CD4⁺ group had to be sacrificed as a result of severe cachexia or high severity scores that developed between day 10 and day 12 post tumour challenge.

Figures 21A and 21B summarize the maximal weight loss and clinical severity scores of the mice from 2 separate experiments. Mice that received F5-TCR + CD3 CD4⁺ T cells developed significantly more weight loss from baseline and higher severity scores than mice that received mock transduced CD4⁺ T cells or F5-TCR CD4⁺ T cells. As a result, no survival benefit was observed following the adoptive transfer of CD4⁺ T cells co-transduced with TCR and

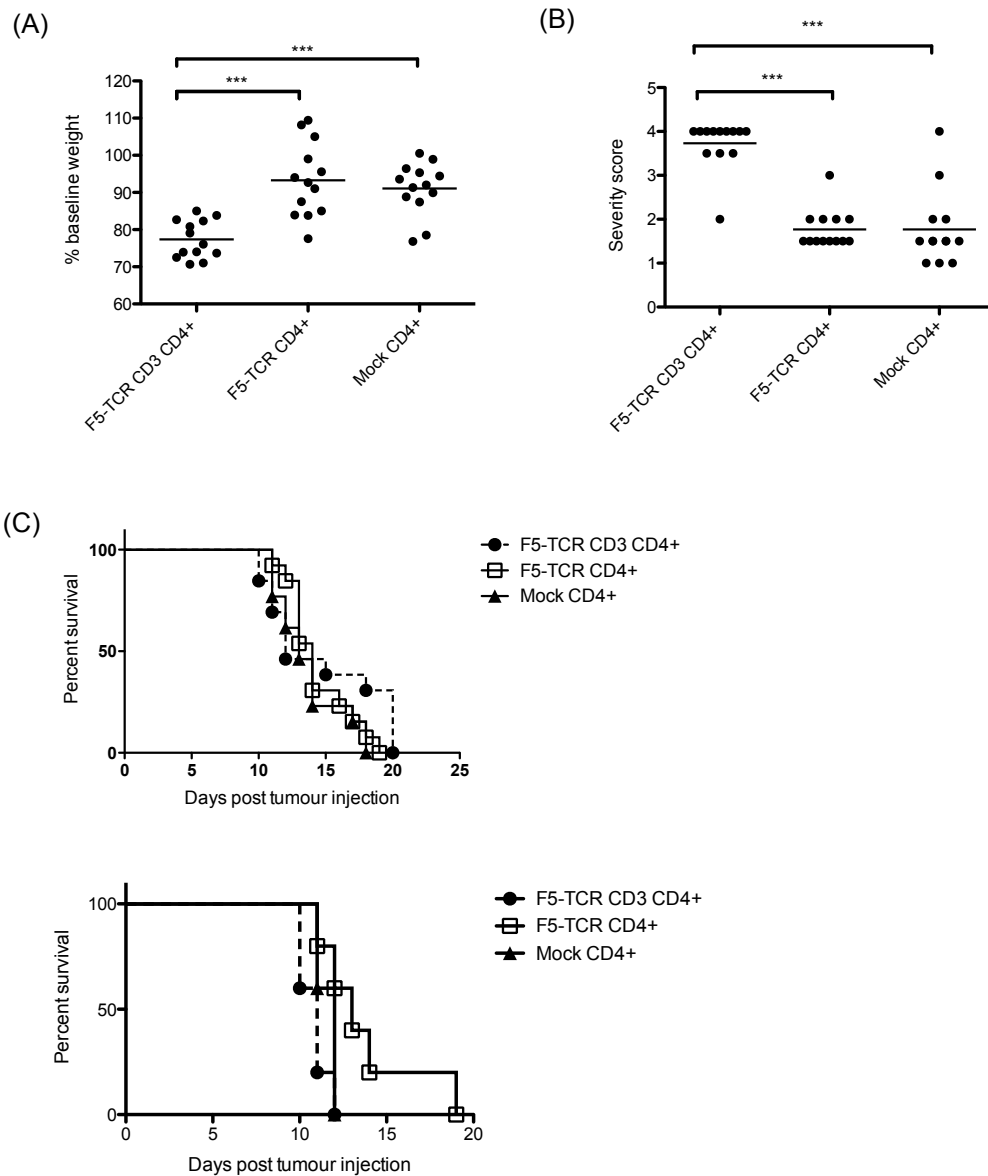


Figure 21 – Adoptive Transfer of F5-TCR + CD3 CD4 T cells leads to marked cachexia and increased severity scores despite tumour protection. (A) Maximal weight loss of recipient mice following adoptive transfer (Results shown are from 2 independent experiments, n= 13 mice) (B) Maximal Severity Scores (C) Kaplan Meier Plots of Survival post tumour challenge in two independent tumour challenge experiments.

CD3 even though there was a reduction in tumour burden in this group (Figure 21C).

Prior to onset of toxicity, adoptively transferred CD4⁺ T cells co-transduced with F5-TCR and CD3 appeared to provide superior tumour protection following adoptive transfer compared to CD4⁺ T cells expressing F5-TCR only. However, no survival benefit was observed due to these marked toxicities.

In this model, an anti-tumour effect was seen in the absence of tumour antigen specific F5-TCR CD8⁺ T cells. Whether this due to direct cytotoxicity of the F5-TCR + CD3 CD4⁺ T cells or whether it is via recruitment of other effector cells to mediate tumour eradication is not clear and was not specifically investigated.

Marked weight loss and increased severity scores were restricted to mice receiving CD4⁺ T cells co-transduced with F5-TCR and additional CD3. No such toxicity was observed in mice who received mock transduced or F5-TCR CD4⁺ T cells. However, all mice in the F5-TCR group died of tumour progression, which may have masked T cell mediated toxicities.

It is possible that the increased TCR expression associated with co-transfer of CD3 increases T cell cross reactivity and targeting of normal tissues by TCR transduced T cells in a non antigen dependent manner. Furthermore, the rate of F5 TCR α and β chain mispairing may be increased with greater TCR

expression levels leading to off target effects due to production of TCR with novel specificities. Mispairing TCR will not have undergone negative selection in the thymus and may not be controlled by peripheral tolerance mechanisms. If mispairing was occurring at a clinically significant level, it may have been expected that toxicity would also have been apparent in the TCR only group but to a lesser degree. It is also possible that transduction of additional CD3 leads to an up regulation of the endogenous TCR. Increased expression of endogenous TCR may itself lead to auto reactivity and the bypassing of normal tolerance mechanisms. In subsequent experiments, the possible mechanisms of toxicity are explored in more detail.

4.4 Adoptive Transfer of F5-TCR + CD3 CD4+ T cells results in enhanced tumour infiltration.

In vitro, F5-TCR + CD3 CD4+ T cells were observed to undergo higher antigen-specific proliferation than F5-TCR only CD4+ T cells and responded to lower concentrations of peptide. Tumour protection was greater with F5-TCR + CD3 CD4+ T cells. It was therefore postulated that F5-TCR + CD3 CD4+ T cells would undergo higher levels of antigen driven expansion in vivo and may also traffic to the tumour site at a faster rate.

To assess T cell trafficking to the tumour site and expansion of transferred cells in vivo, bioluminescence imaging of luciferase positive TCR-transduced CD4+ T cells was utilized. CD4+ T cells obtained from luciferase+ transgenic C57BL/6 mice were used for TCR transduction and adoptive transfer. In vivo

tumour challenge with luciferase negative EL4-NP tumour cells was administered. Recipient mice were sub lethally irradiated with 5.5 Gy irradiation on day 0 and 4 hours subsequent to this injected with 1×10^6 EL-NP luciferase negative tumour cells. On Day 1 post tumour challenge, mice were injected intravenously with 1×10^6 luciferase positive F5-TCR CD4+ T cells, 1×10^6 F5-TCR + CD3 CD4+ T cells or 1×10^6 mock transduced CD4+ T cells. Infiltration of CD4+ T cells into the tumour site was visualized serially post tumour challenge using in vivo bioluminescence imaging. F5-TCR + CD3 CD4+ T cells were first detectable in the tumour site at seven days post tumour challenge. TCR only CD4+ T cells could be detected at the tumour site but not until nine days post tumour challenge. (Figure 22A).

A control C57BL/6 mouse was injected subcutaneously with different doses of luciferase+ T cells, and the bioluminescent signal achieved from each T cell dose was measured (Figure 22D). (These T cell titration measurements were performed in conjunction with Doctor Maryam Ahmadhi). The bioluminescent signal from each mouse at each time point was converted into an approximate number of luciferase+ T cells. The mean bioluminescent signal in photon/sec and mean T cell number are shown in Figures 22B and 22C. CD4+ T cells co-transduced with F5-TCR and CD3 were detectable in significantly greater numbers at the tumour site at both day 7 and day 9 post adoptive transfer compared to F5-TCR only CD4+ T cells ($p \leq 0.01$). There was no significant increase in expansion of F5-TCR only CD4+ T cells when compared to mock transduced CD4+ T cells. These experiments indicated that F5-TCR + CD3 CD4+ T cells were able to expand more efficiently in vivo following adoptive

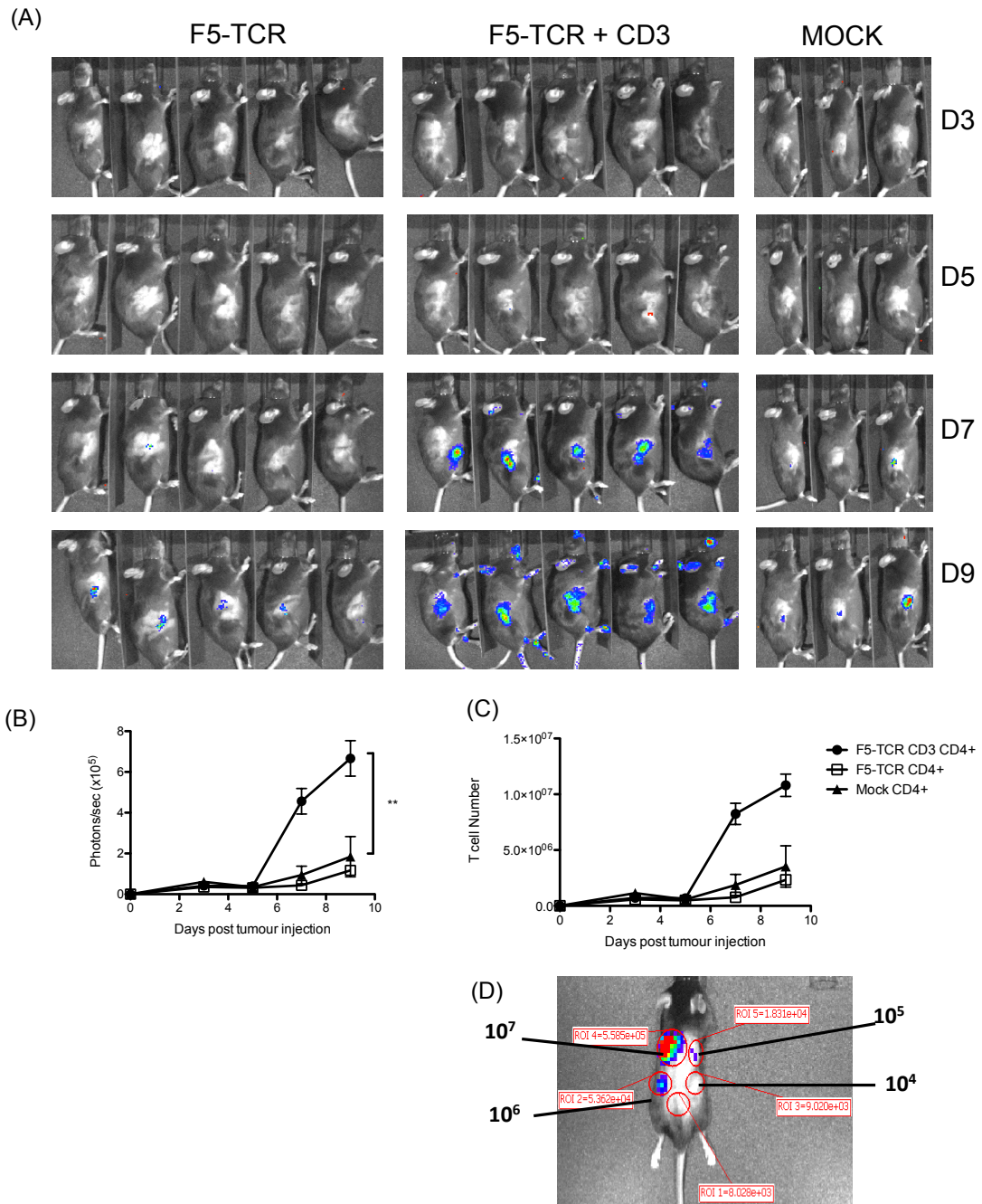


Figure 22 – Enhanced tumour infiltration by F5-TCR + CD3 CD4+ T cells. C57BL/6 Thy 1.2 donor mice received sub lethal irradiation with 5.5 Gy on day 0 and 4 hours later received 1×10^6 EL4-NP tumour cells subcutaneously. On Day 1, mice received 1×10^6 F5-TCR, F5-TCR + CD3 or mock transduced CD4+ luciferase+ T cells intravenously. Results shown are from one experiment with 5 mice per group. (A) Bioluminescent signal demonstrating infiltration by adoptively transferred CD4+ T cells. (B) Mean bioluminescent signal in photons/second recorded at tumour site (C) Photons/second were converted into an approximate T cell number at tumour site. Mean T cell number is shown. (D) Bioluminescent signal seen following injections of defined luciferase+ T cell number.

transfer and were able to traffic to the tumour site more effectively than F5-TCR only CD4+ T cells.

Infiltration of F5-TCR + CD3 CD4+ T cells in peripheral tissues such as the nose, tail and paws of the mice was also observed. It is hypothesized that the TCR transduced T cells are binding to an antigen in the periphery which is distant from the site of the original injected tumour and is unlikely to represent metastatic tumour given the location. Thus the TCR transduced T cells are recognizing a peripheral antigen that is not NP peptide which lends support to the hypothesis that F5-TCR + CD3 CD4+ T cells may be autoreactive or cross-reactive, assuming the cognate antigen is only expressed in the tumour tissue.

4.5 In vivo expansion and persistence is enhanced following adoptive transfer of F5-TCR CD3 CD4+ T cells

To assess the expansion and persistence of TCR-transduced CD4+ T cells post adoptive transfer, the transferred T cells were isolated and quantified from different organs. Tissue samples were obtained from bone marrow, lymph node and spleen on the day of sacrifice. The results shown were from 4 independent experiments with a total of 22 mice per group. Single cell suspensions from each organ were prepared as described in the section 2.7 and stained with anti-murine Thy 1.1 and anti murine CD4 monoclonal antibodies prior to FACS analysis. As recipient mice were Thy 1.2+, all of the

Thy 1.1+ CD4+ T cells detected were derived from the adoptively transferred cells.

Following adoptive transfer, a higher proportion of Thy 1.1+ CD4+ T cells were detectable in mice that received F5-TCR + CD3 CD4+ T cells compared to mice given F5-TCR only CD4+ T cells. A significantly higher percentage of adoptively transferred T cells were seen in the F5-TCR + CD3 group in all organs analyzed. In mice that received TCR only CD4+ T cells, the mean percentage of Thy 1.1+ CD4+ T cells (the percentage of viable lymphocytes that were Thy 1.1+ CD4+) detectable within the bone marrow, spleen and lymph node was 2.2% (range 0.02-11.4%), 4.5% (range 0.04%-24%) and 5.7% (range 0.01-19.6%) respectively. In mice that received CD4+ T cells co-transduced with F5-TCR and CD3 the mean percentage of Thy 1.1+ CD4+ T cells detected within bone marrow, spleen and lymph node was 28.9% (range 1.1-75.9%)($p \leq 0.001$), 18.8% (range 0.9-53.4%)($p \leq 0.001$) and 26.5% (range 0.6%-75.5%) ($p \leq 0.001$) respectively (Figure 23). When the absolute number of CD4+ Thy 1.1 T cells were analyzed there was a significantly higher number of adoptively transferred cells detected in the bone marrow and spleen of mice that received F5-TCR + CD3 CD4+ T cells compared to F5-TCR CD4+ T cells. Within the spleen, there was no significant difference in absolute cell number of CD4+ Thy1.1+ T cells seen between the two groups.

In this analysis, the survival time of mice post tumour challenge ranged from 10 – 25 days. With increasing time post irradiation, re-expansion of recipient haematopoiesis will be greater thus reducing the detectable proportion of

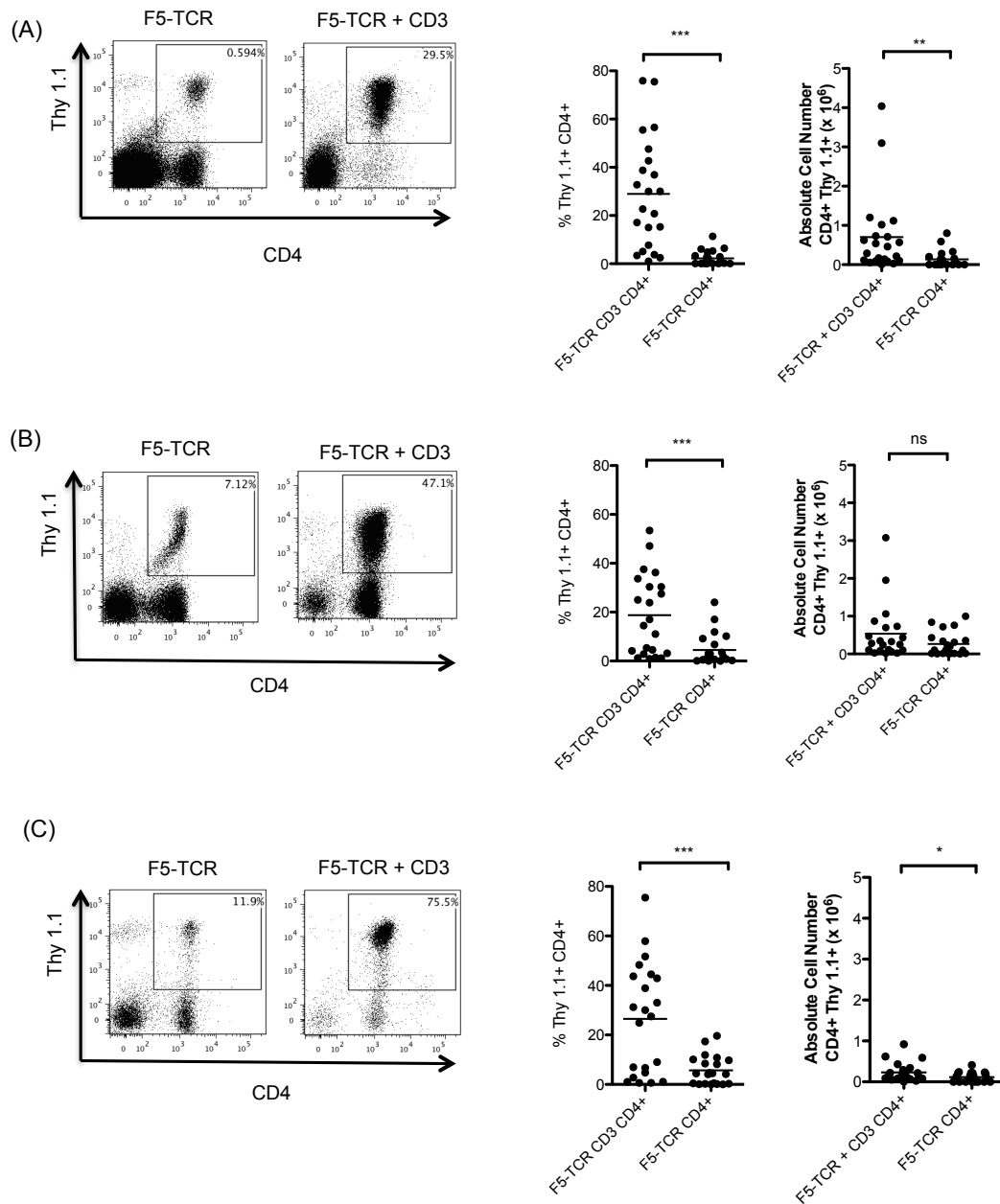


Figure 23 . In vivo expansion and persistence of F5-TCR CD4+ T cells is enhanced by the presence of additional CD3. Analysis of bone marrow, spleen and lymph node samples to assess persistence/expansion of F5-TCR or F5-TCR + CD3 TCR transduced CD4+ T cells following tumour challenge experiments. Samples were taken on day 10-25 post tumour challenge. Cells were gated on viable lymphocytes and representative FACS plots of Thy 1.1+ CD4+ T cells and % of Thy 1.1+ CD4+ T cells and absolute cell number in (A) bone marrow, (B) spleen and (C) lymph node are shown. Plots show results from 4 independent experiments.

adoptively transferred T cells. Differences of cell expansion seen between the F5-TCR only and F5-TCR + CD3 groups may be exaggerated, particularly if there is a bias to one group being analyzed at much earlier time points. In order to reduce this bias, a comparison of mean percentage of Thy 1.1+ and CD4+ T cells was performed on samples taken on day 10-14 post tumour challenge (n=13/group) and samples taken on day 15-25 post tumour challenge (n=8/group). In the analysis performed on day 10-14 there was a significantly higher percentage of Thy1.1+ CD4+ T cells in the bone marrow ($p \leq 0.01$), spleen ($p \leq 0.01$) and lymph node ($p \leq 0.01$) in mice that received F5-TCR + CD3 CD4+ T cells compared to mice that received F5-TCR only CD4+ T cells (Figure 24). This would suggest that F5-TCR + CD3 CD4+ T cells underwent a higher level of in vivo expansion initially following adoptive transfer compared to F5-TCR only CD4+ T cells.

Analysis of later time points reflects more the ability of transferred T cells to persist in vivo rather than initial expansion. In the analysis performed on samples taken between day 15 and day 25 (n=8/group) there was still a significantly higher proportion of Thy 1.1+ CD4+ T cells present within the bone marrow ($p \leq 0.05$) and spleen ($p \leq 0.01$) of mice that received F5-TCR + CD3 CD4+ T cells. However, persistence of adoptively transferred CD4+ T cells within the lymph nodes is not significantly different following transfer of F5-TCR + CD3 CD4+ or F5-TCR only CD4+ T cells. This may suggest that F5-TCR + CD3 CD4+ T cells persist more readily within the bone marrow and spleen than in the lymph node or they may have undergone initial greater expansion within these organs. This may also reflect more effective

persistence of F5-TCR CD4⁺ T cells within the lymph node than within other organs. Figure 24 and 25 shows the analysis of the two different time points and also summarizes the minimum, maximum and means of the populations.

Whilst these experiments assume that the superior expansion and persistence of F5-TCR + CD3 CD4⁺ T cells is secondary to recognition of cognate NP peptide, expansion may also be driven by recognition of a self antigen as a result of increased auto reactivity of the F5-TCR + CD3 CD4⁺ T cells. It may also reflect expansion of CD4⁺ T cells that are expressing mispaired TCR which have unknown specificities.

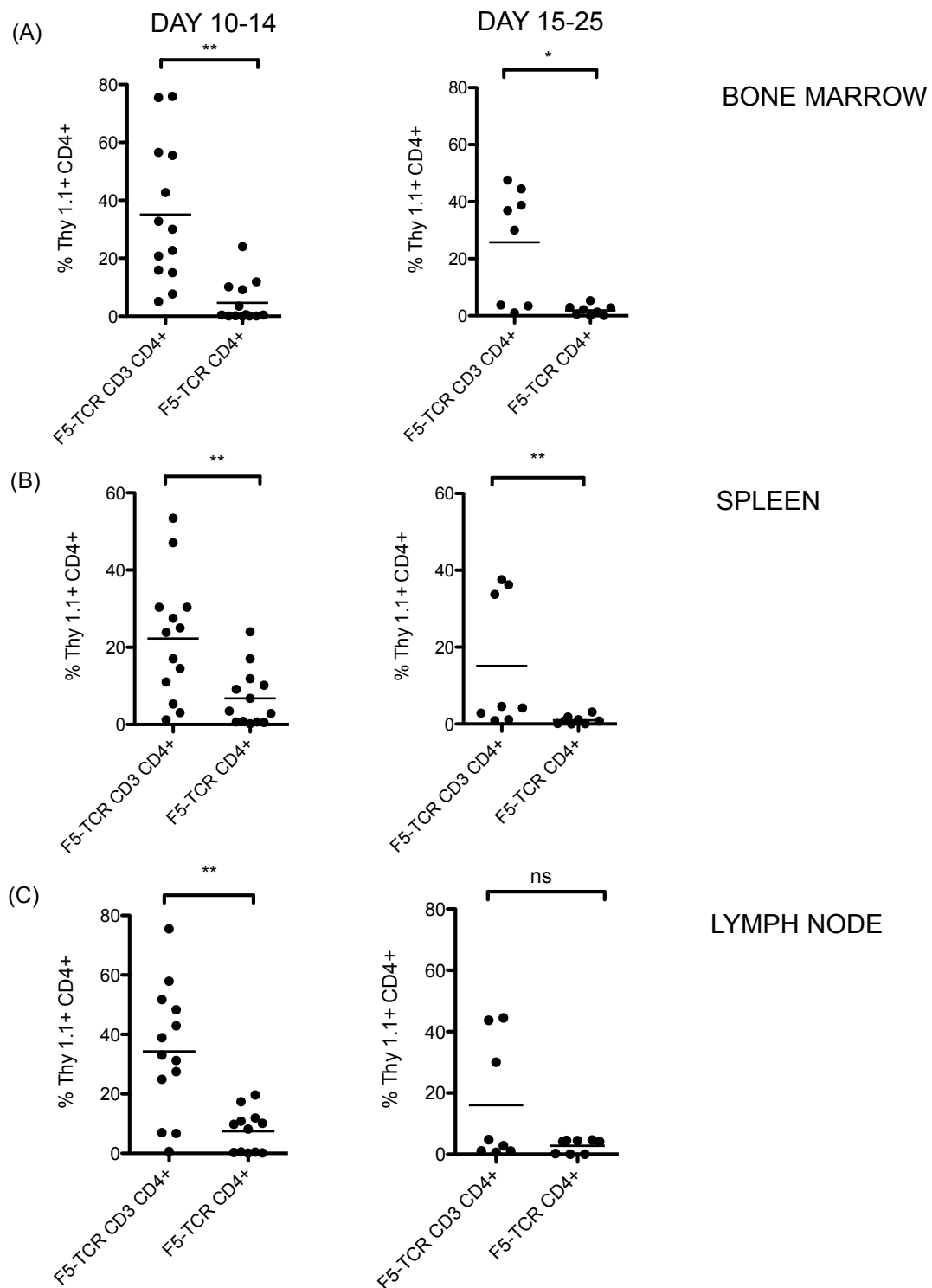


Figure 24 – Persistence of F5-TCR only and F5-TCR + CD3 T cells at early and late time points post adoptive transfer into tumour bearing mice. Percentage of Thy 1.1+ and CD4+ T cells were compared at day 10-14 and day 15-25 in (A) Bone Marrow (B) Spleen and (C) Lymph Node.

BONE MARROW (%Thy 1.1+ CD4+)	F5-TCR CD4+ (Day10-14)	F5-TCR CD3 CD4+ (Day10-14)	F5-TCR CD4+ (Day15-25)	F5-TCR CD3 CD4+ (Day15-25)
Minimum	0.02	5.13	0.08	1.06
Maximum	24	75.9	5.3	47.6
Mean	4.6	35	1.88	25.76

SPLEEN (%Thy 1.1+ CD4+)	F5-TCR CD4+ (Day10-14)	F5-TCR CD3 CD4+ (Day10-14)	F5-TCR CD4+ (Day15-25)	F5-TCR CD3 CD4+ (Day15-25)
Minimum	0.2	1.2	0.04	0.9
Maximum	24	53.4	3.12	37.6
Mean	6.8	22.3	0.99	15.1

LYMPH NODE (%Thy 1.1+ CD4+)	F5-TCR CD4+ (Day10-14)	F5-TCR CD3 CD4+ (Day10-14)	F5-TCR CD4+ (Day15-25)	F5-TCR CD3 CD4+ (Day15-25)
Minimum	0.09	0.6	0.01	0.6
Maximum	19.6	75.5	4.6	44.5
Mean	7.9	34.3	2.8	16

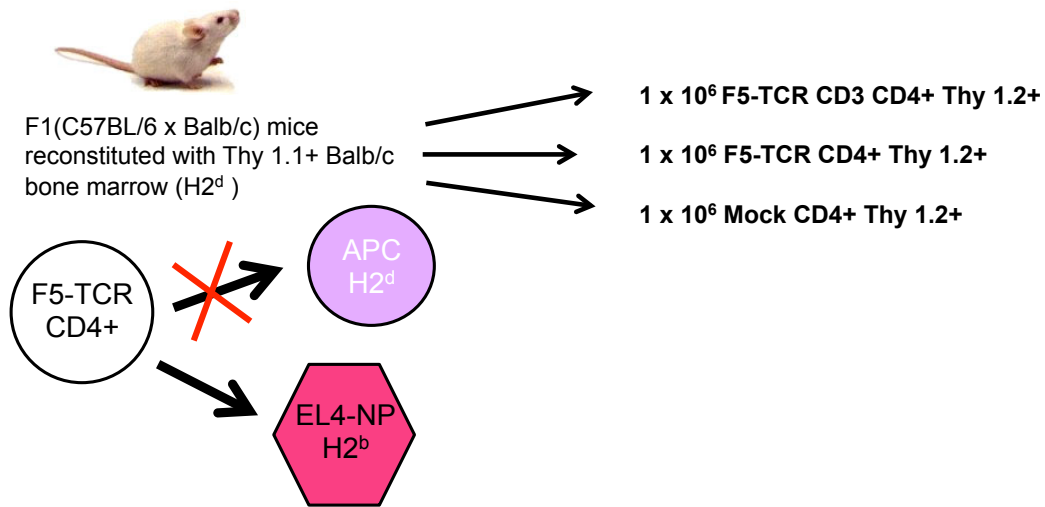
Figure 25 – Summary of Minimum, Maximum and Mean % Thy 1.1+ CD4+ T cells at Day 10-14 and Day 15-25.

4.6 CD4⁺ T cells expressing class I restricted TCR recognize class II negative tumour cells in vivo.

CD4⁺ T cells expressing a class I restricted TCR recognize peptide presented by class I MHC molecules. It has not been established whether class I restricted CD4⁺ T cells can respond to peptide-MHC-I on the surface of any nucleated cell or whether they can only respond to peptide-MHC-I presented by professional APCs. The in vitro data described in chapter 3 supports the hypothesis that CD4⁺ T cells expressing the class I restricted TCR, F5-TCR can be stimulated by antigen that is directly presented by class I MHC on tumour cells (which lack class II MHC).

To explore this further in vivo, a tumour challenge was carried out in a model system using bone marrow chimeric mice in which the haematopoietic cells of the recipient mice were of a different H2 haplotype to that which is recognized by the F5-TCR. In this context, any antigen specific responses would be mediated by direct recognition of peptide-MHC-I presented by EL4-NP tumour cells and not by presentation of NP peptide by class I MHC on professional APCs. The experimental model is shown schematically in Figure 26. The F5-TCR recognizes the NP peptide in the context of H2-K^b. This model system used bone marrow cells derived from C57BL/6 mice (H2^b) or bone marrow cells derived from Balb/c mice (H2^d). In bone marrow chimeric mice reconstituted with Balb/c (H2^d) haematopoietic cells, F5-TCR CD4⁺ T cells can only respond to NP peptide presented by H2^b on EL4-NP tumour cells and not by professional APCs which express H2^d. Therefore if tumour specific

(A) [Balb/c→F1] chimeras



(B) [C57BL/6→F1]chimeras

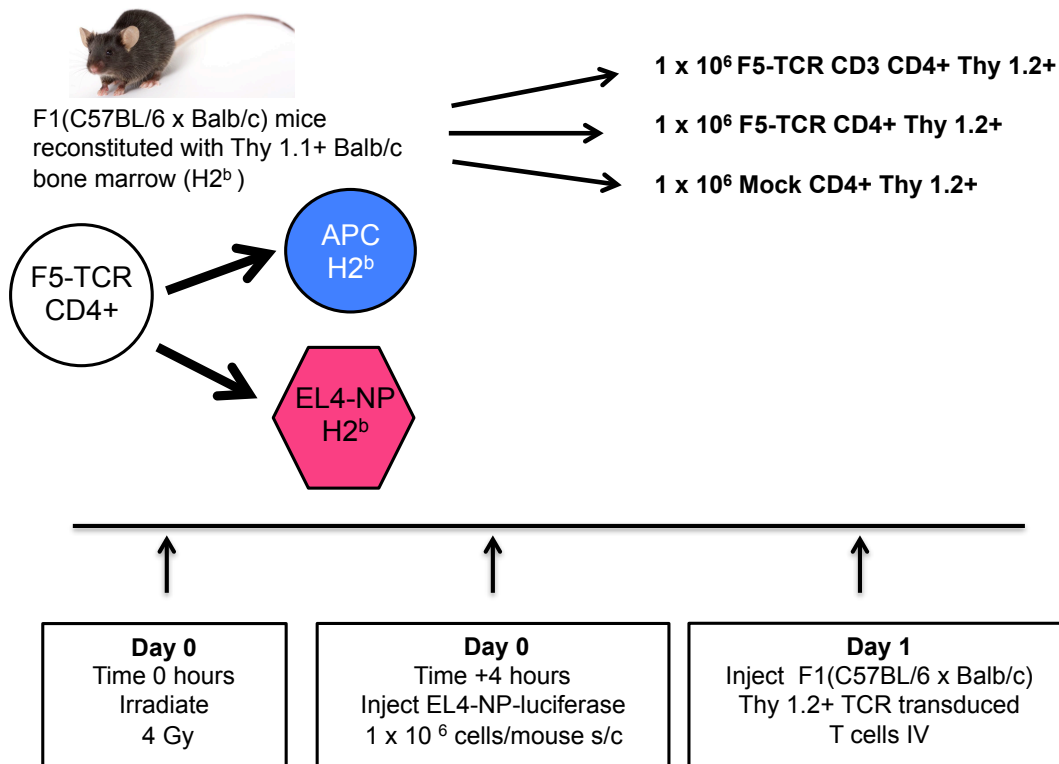


Figure 26 – Schematic representation of experimental set up for bone marrow chimeric tumour challenge experiments.

responses were seen in the Balb/c chimeric mice it would support the hypothesis that class I restricted CD4⁺ T cells could respond directly to antigen presented by a non professional APC.

To produce bone marrow chimeric mice, F1 (C57BL/6 x Balb/c) mice were irradiated and then given a bone marrow transplant with T cell depleted Thy 1.1⁺ Balb/c bone marrow cells or Thy 1.1⁺ C57BL/6 bone marrow cells. Mice were then housed in pathogen free conditions for 12 weeks to allow time for full haematopoietic reconstitution. Prior to tumour challenge experiments, blood samples from the chimeric mice were obtained and analyzed to assess full donor chimerism by staining for CD19, CD4, CD8, CD11b, H2-K^b and H2-K^d (Figure 27 and 28). Splenocytes derived from Thy 1.2⁺ F1 (C57BL/6 x Balb/c) mice were used for transduction and adoptive transfer so that the adoptively transferred cells could be monitored ex vivo.

For the tumour challenge experiments a lower irradiation dose was used as these mice had previously been irradiated with 2 x 5.5 Gy at the time of bone marrow transplant. In addition Balb/c bone marrow cells are more radiosensitive than C57BL/6 bone marrow cells. Recipient mice were irradiated with 4 Gy on day 0 and then 4 hours post irradiation were injected subcutaneously with EL4-NP Luciferase⁺ tumour cells. On day 1, recipient mice were injected IV with 1 x 10⁶ F5-TCR + CD3 CD4⁺ T cells, F5-TCR CD4⁺ T cells or mock transduced CD4⁺ T cells. Mice were monitored for tumour growth and with bioluminescent imaging. Mice were monitored for weight loss and severity scoring.

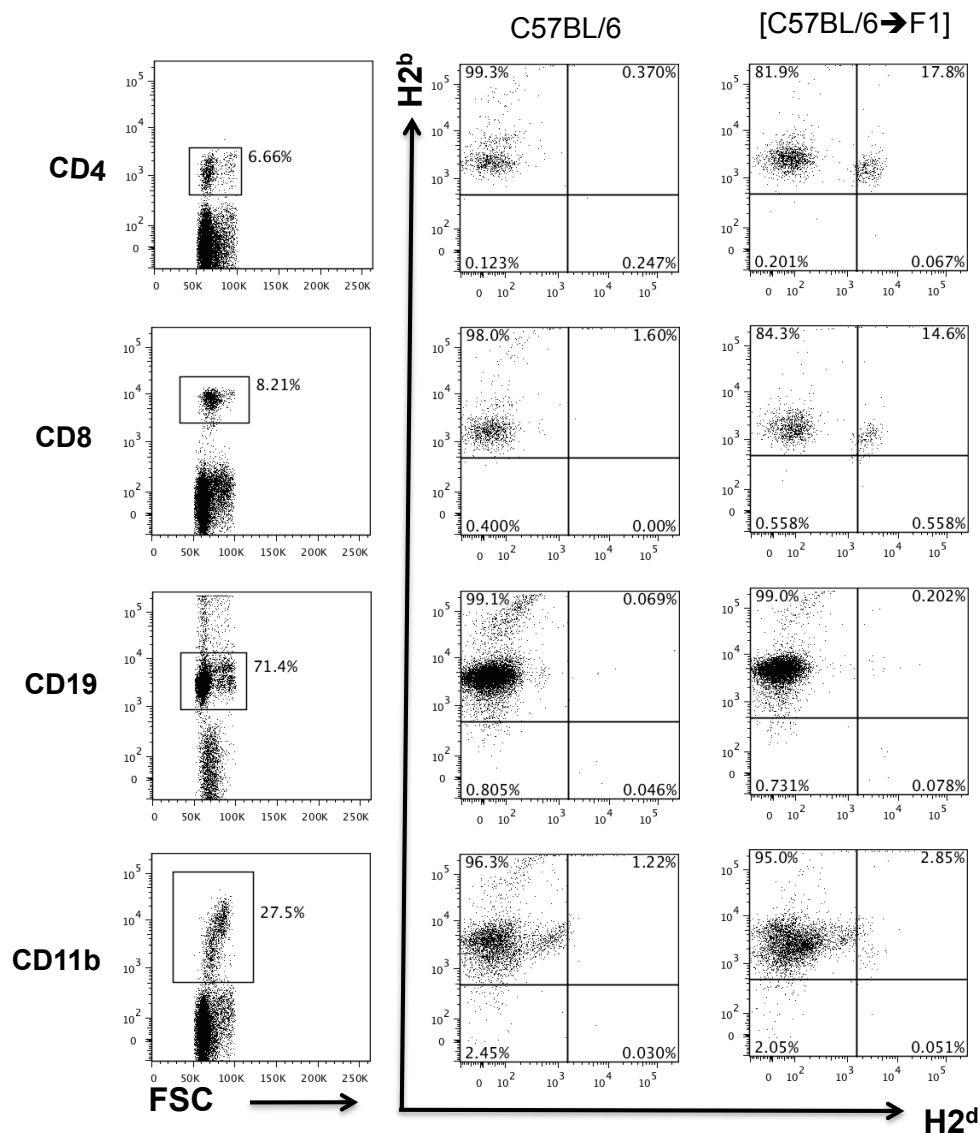


Figure 27 – Analysis of engraftment of C57BL/6→F1 chimeric mice. Representative plots of blood samples taken from bone marrow chimeric mice on day -1 of tumour challenge experiments (3 months post initial establishment of chimeras.) F1(C57BL/6 x Balb/c mice) were lethally irradiated and then given a bone marrow transplant with CD4/8 depleted C57BL/6 (H2^b) bone marrow. Plots show the extent of chimerism in C57BL/6 mice and [C57BL/6→F1] chimeras in the CD4+, CD8+, CD19+ and CD11+ populations. For FACS analysis cells were initially gated on viable lymphocytes then gated on CD4, CD8, CD19 or CD11+ cells as shown and expression of H2^b versus H2^d was analyzed in each population.

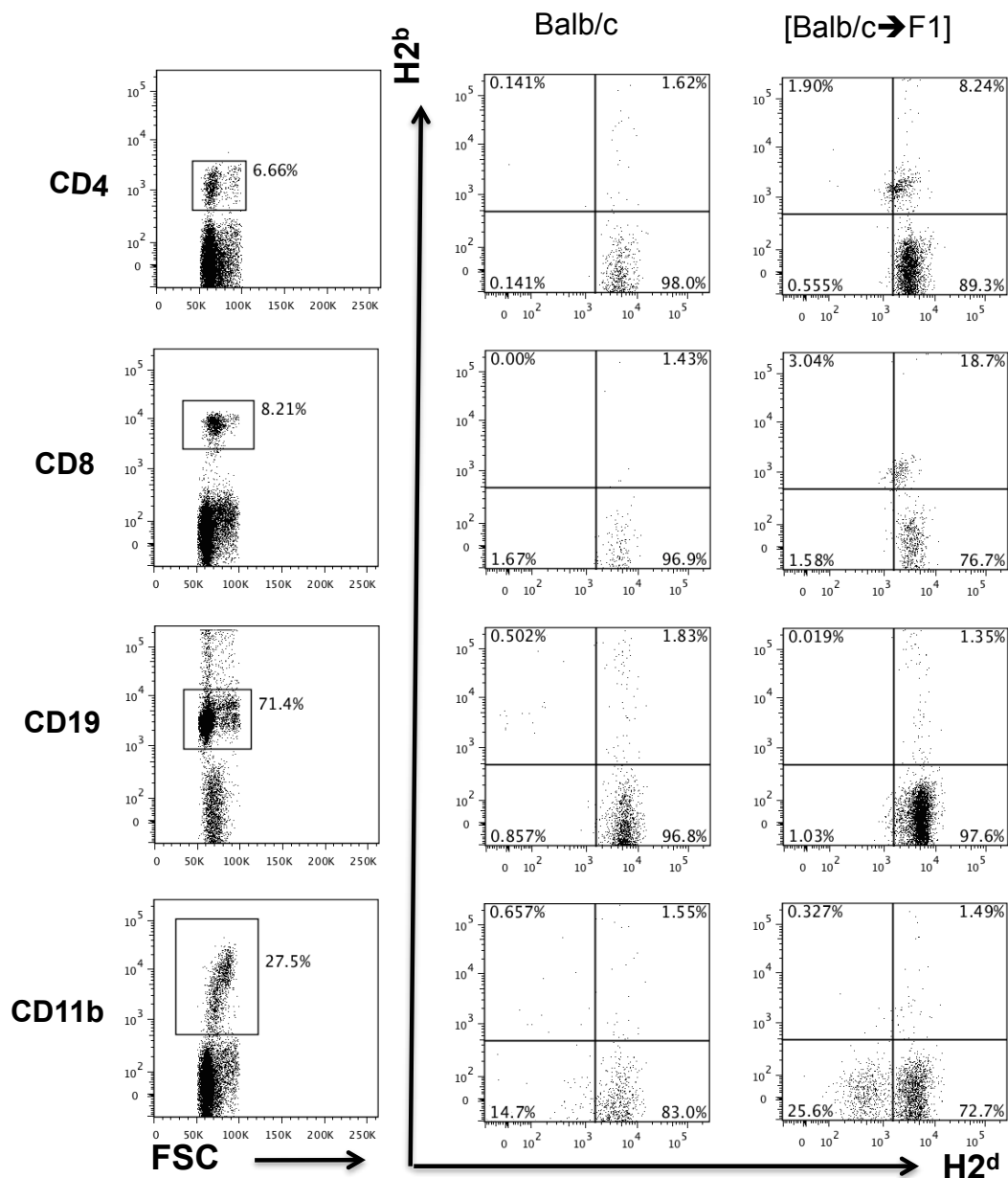


Figure 28 – Analysis of engraftment of Balb/c→ F1 chimeric mice. Representative plots of blood samples taken from bone marrow chimeric mice on day -1 of tumour challenge experiments (3 months post initial establishment of chimeras.) F1(C57BL/6 x Balb/c mice) were lethally irradiated and then given a bone marrow transplant with CD4/8 depleted Balb/c (H2^d) bone marrow. Plots show the extent of chimerism in Balb/c mice and [Balb/c→ F1] chimeras in the CD4+, CD8+, CD19+ and CD11+ populations. For FACS analysis cells were initially gated on viable lymphocytes then gated on CD4, CD8, CD19 or CD11+ cells as shown and expression of H2^b versus H2^d was analyzed in each population.

In the {C57BL/6→F1} chimeras, adoptive transfer of F5-TCR + CD3 CD4⁺ T cells resulted in more efficient control of tumour growth than F5-TCR CD4⁺ T cells. In the F5-TCR + CD3 CD4⁺ group, tumour growth peaked at day 16 post tumour challenge and then tumour regression was seen in all mice. No mice died of progressive tumour growth. In the F5-TCR only CD4⁺ group, tumour burden was higher and peaked at day 19 post tumour challenge. 3/5 mice died of progressive tumour growth. No mice in either group died of toxicity which likely reflects the lower irradiation dose that was used in this experiment (Figure 29).

In the model using {Balb/c→F1} chimeric recipients, the transduced T cells were not able to respond NP peptide presented by H2^d professional APCs and thus tumour eradication is dependent on direct presentation by the EL4-NP tumour cells. Following the adoptive transfer of F5-TCR + CD3 CD4⁺ T cells into {Balb/c→F1} chimeras, minimal tumour growth was seen in 4/5 mice. Only 1/5 mice had a measurable tumour burden that peaked at 20mm² on day 20 post tumour challenge. None of the mice that received F5-TCR + CD3 CD4⁺ T cells had progressive tumour growth. Following adoptive transfer of F5-TCR CD4⁺ T cells into {Balb/c→F1} chimeras, the tumour burden was higher than that seen in the F5-TCR + CD3 CD4⁺ group although this was not a significant difference. In the F5-TCR CD4⁺ group, 1/5 mice cleared the tumour completely and one mouse died of tumour progression (Figure 30). These results (and the in vitro data) would suggest that CD4⁺ T cells expressing a class I restricted TCR can recognize EL4-NP tumour cells directly even in the absence of interaction with professional APCs.

C57BL/6→F1 chimera recipient mice

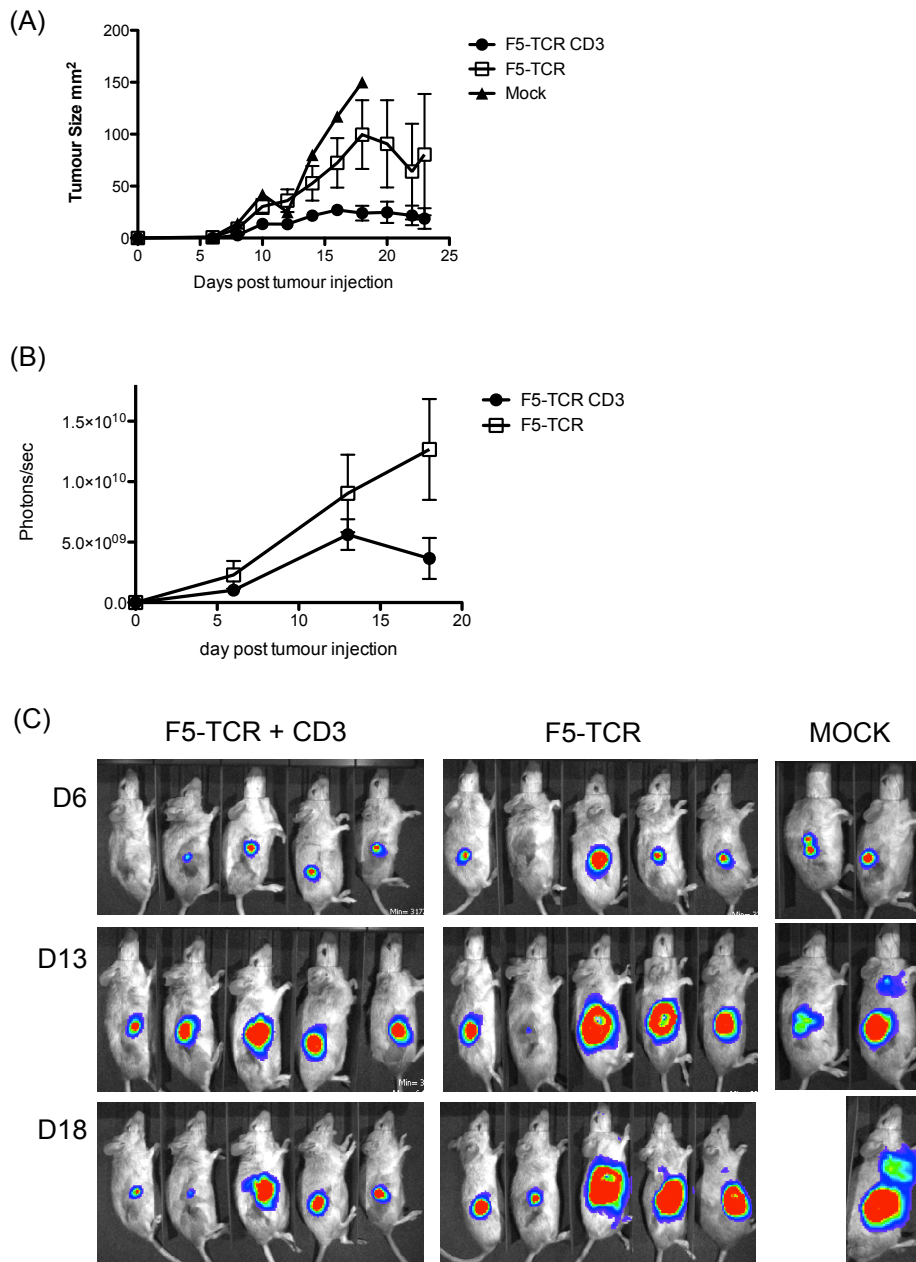


Figure 29 – CD4⁺ T cells expressing class I restricted TCR can respond to antigen presented by non professional antigen presenting cells. F1 (C57BL/6 x Balb/c) recipient mice which had been reconstituted with Thy 1.1⁺ C57BL/6 bone marrow were irradiated on day 0 with 4 Gray and then 4 hours later injected subcutaneously with 1×10^6 EL4-NP luciferase + tumour cells. Mice were injected IV with 1×10^6 F5-TCR CD3 CD4⁺, F5-TCR CD4⁺ or mock transduced CD4⁺ T cells on day 1. (A) Mean Tumour Area measured by calipers. (B) Mean bioluminescent signal in photons/second recorded at site of tumour growth (C) Bioluminescent signal at tumour site measured on day 6-18 post tumour challenge.

Balb/c→F1 chimera recipient mice

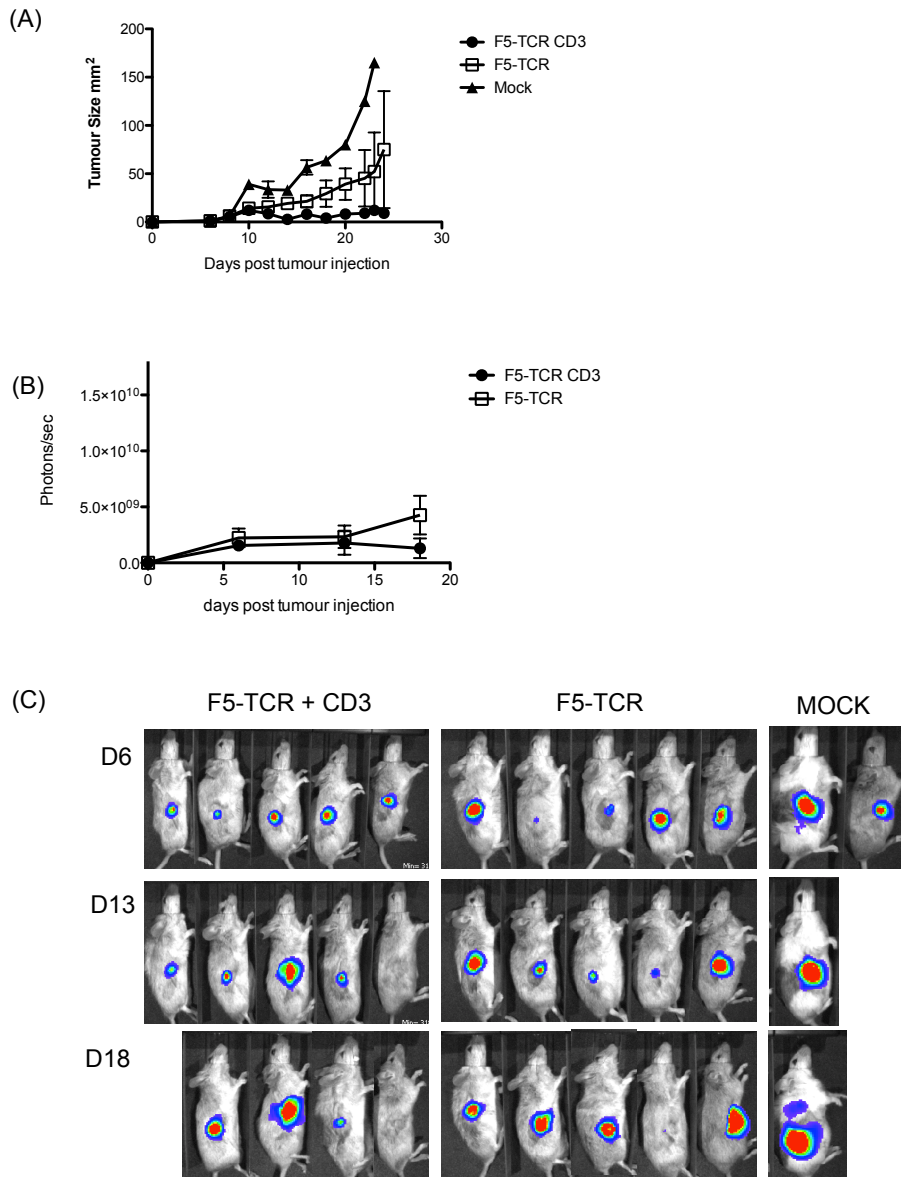


Figure 30 – CD4⁺ T cells expressing class I restricted TCR can respond to antigen presented by non professional antigen presenting cells. F1(C57BL/6 x Balb/c) recipient mice which had been reconstituted with Thy 1.1+ Balb/c bone marrow were irradiated on day 0 with 4 Gy and then 4 hours later injected subcutaneously with 1×10^6 EL4-NP luciferase + tumour cells. Mice were injected IV with 1×10^6 F5-TCR CD3 CD4⁺, F5-TCR CD4⁺ or Mock Transduced CD4⁺ T cells on day 1. (A) Mean tumour area measured by calipers. (B) Mean bioluminescent signal in photons/second recorded at site of tumour growth(C) Bioluminescence signal at tumour site measured on day 6-18 post tumour challenge.

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In addition, these results also suggest that tumour eradication was more efficient in the {Balb/c→F1} chimera model where antigen was presented solely by non-professional APC eg the EL4-NP tumour cell. These results were however complicated by toxicity seen in {Balb/c→F1} chimeras following adoptive transfer of transduced CD4⁺ T cells. In the F5-TCR + CD3 CD4⁺ group, 4/5 mice were culled before the end of the experiment because of marked weight loss or high severity score on day 14,17 and 20 post tumour challenge. In the F5-TCR only CD4⁺ group, 2/5 mice were culled before the end of the experiment because of toxicity on day 17 and day 20 post tumour challenge. None of the {C57BL/6→F1} chimera recipients died of toxicity. These differences may reflect the increased sensitivity of the {Balb/c→F1} chimeras to the effects of the 4Gy irradiation compared to the {C57BL/6→F1} chimeras or may reflect increased targeting of normal tissues by the adoptively transferred T cells in the {Balb/c→F1} chimeras.

When looking at the percentage of Thy 1.2⁺ CD4⁺ T cells within the bone marrow, spleen and lymph nodes the results have to be interpreted with care as there were only small numbers and the mice were culled at different time points post tumour challenge. (day 14 n=1; day 17 n=2; day 19 n=1; day 20 n=4; day 23 n=1; day 24 n=1; day 26 n=10). No significant differences were observed between any of the groups or in any of the organs analyzed (Figure 31). The {Balb/c→F1} chimeras receiving F5-TCR + CD3 CD4⁺ T cells had the highest percentage of adoptively transferred cells in all organs whilst the

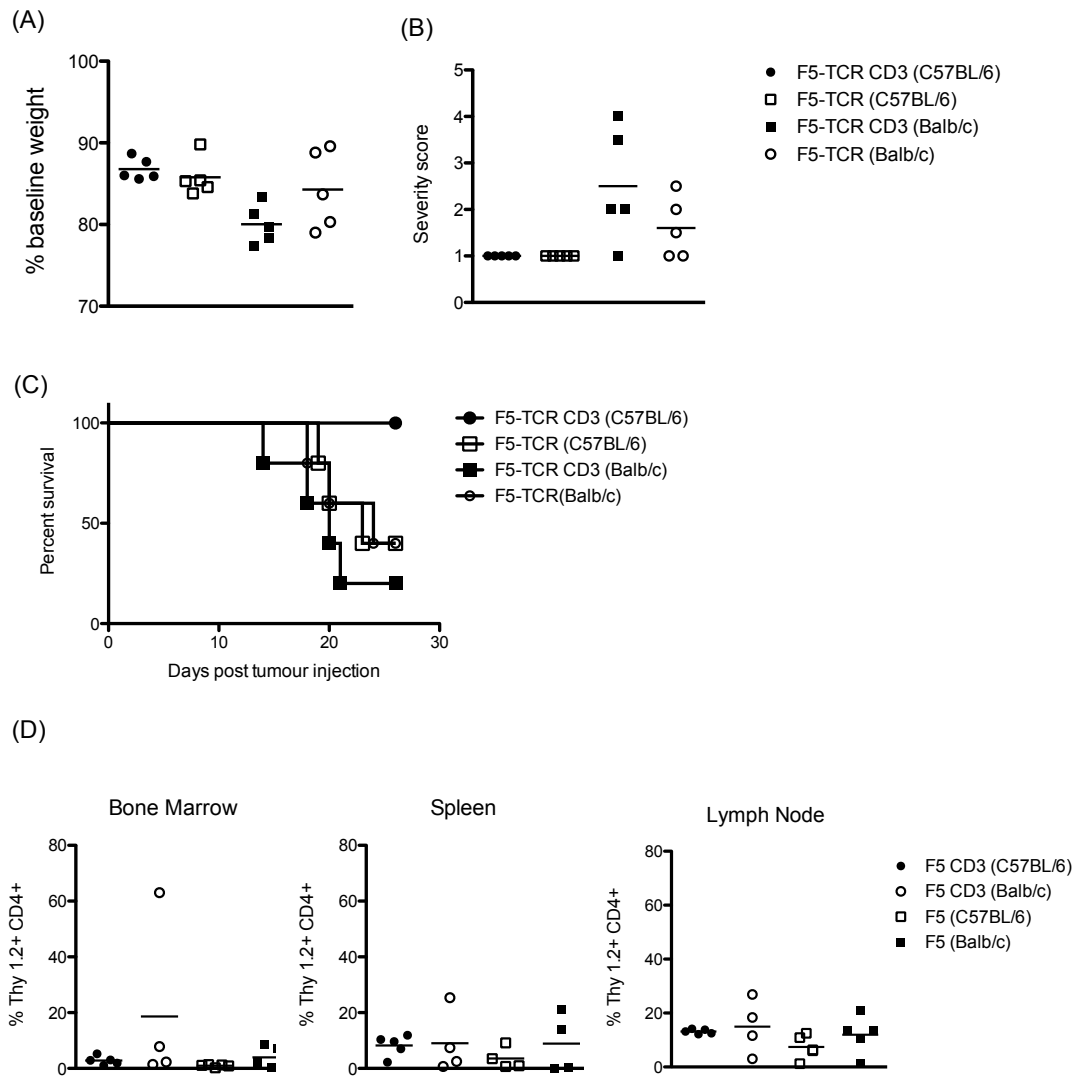


Figure 31 – CD4⁺ T cells expressing class I restricted TCR can respond to antigen presented by non professional antigen presenting cells. (A) Maximal weight loss (B) Maximal severity score (C) Kaplan Meier plot of Overall Survival. (D) Mean percentage of Thy 1.2⁺ CD4⁺ T cells in bone marrow, lymph node and spleen.

{C57BL/6→F1} chimeras receiving F5-TCR CD4⁺ T cells had the lowest percentage of adoptively transferred populations at the time of analysis. Thus F5-TCR or F5-TCR + CD3 CD4⁺ T cells that have been adoptively transferred into recipients where the APCs are of a different MHC background to that recognized by the F5-TCR can persist and expand to the same or higher levels than seen in recipients with APCs expressing the correct MHC type. Combined with the tumour growth curves this adds support to the fact the F5-TCR expressing CD4⁺ T cells can respond to tumour that is presented by the tumour cells directly and don't have an absolute requirement for the presence of a professional APC.

4.7 Discussion

There is now increasing evidence that CD4⁺ T cells can provide tumour protection in vivo by direct cytotoxicity to tumour cells or by provision of help to tumour specific CD8⁺ T cells and to innate immune cells. The efficiency of CD4⁺ T cells at reducing tumour burden has been effectively demonstrated using adoptive transfer of class II restricted transgenic CD4⁺ T cells. The adoptive transfer of as few as 50,000 transgenic CD4⁺ T cells specific for TRP1⁺ (a melanoma differentiation antigen) could be used to eradicate advanced melanoma in a murine model (269). These adoptively transferred CD4⁺ T cells had a Th1 phenotype, producing large amounts of TNF- α , IFN- γ and IL2 and expressed high levels of granzyme and demonstrated in vivo cytotoxicity. Cytotoxicity of the adoptively transferred CD4⁺ TRP1⁺ cells was

mediated via direct recognition of class II MHC on the tumour cells and dependent on degranulation of granzyme containing lytic granules. Production of IFN- γ by the adoptively transferred CD4⁺ T cells led to upregulation of class II MHC on the tumour cell. Tumour rejection was also independent of the endogenous CD4⁺, CD8⁺, B cell and NK cell populations and of endogenous perforin killing activity.

In some models it has been suggested that CD4⁺ T cells may provide even more efficient tumour protection than CD8⁺ T cells. Kerkar et al transduced murine CD8⁺ T cells with a class I restricted TCR specific for melanoma antigen, gp100 and transduced murine CD4⁺ T cells with a class II restricted TCR specific for TRP-1. To test function, transduced T cells were adoptively transferred into a B16 murine melanoma model. Interestingly the adoptive transfer of gp100-CD8⁺ T cells led to delayed tumour growth whilst the adoptive transfer of TRP-1 transduced CD4⁺ T cells led to marked anti-tumour immunity. This could not be explained by a increased expression level of the TCR within the CD4⁺ population as the adoptive transfer of a five fold higher number of CD8⁺ T cells did not lead to enhanced anti-tumour responses. It was thought that these differences were either as a result of inherent anti-tumour immunity provided by the 2 differing populations of cells or due to some intrinsic difference in the individual TCR properties (287).

The main advantage of using CD4⁺ T cells transduced with a class I restricted TCR is that it obviates the need for provision of separate class II restricted epitopes which are less frequently expressed by cancer cells and also difficult

to define. In addition CD8⁺ and CD4⁺ T cells could be transferred that both target the same tumour antigen or could be transduced with different class I restricted TCR to target to separate tumour epitopes. It may be desirable too target different tumour antigens as this may promote determinant spreading and reduces the risk of development of antigen loss variants. Whether class I restricted CD4⁺ T cells are as effective as cytotoxic CD8⁺ T cells expressing the same TCR or as effective as class II restricted CD4⁺ T cells at providing in vivo help or direct cytotoxicity has not yet been clearly delineated.

The clinical and preclinical data using class I restricted CD4⁺ T cells is still quite limited and the exact mechanisms by which they mediate anti-tumour immunity are still being clarified. There have been a number of papers now demonstrating that class I restricted CD4⁺ T cells can provide anti tumour protection in vivo, either alone or in conjunction with tumour specific CD8⁺ T cells. Frankel et al (288) used a TCR derived from a TIL specific for a melanoma epitope, tyrosinase 368-376. Unusually, the TIL was unique in that it was reactive to HLA-A2 but it was expressed by a CD4⁺/CD8⁻ T cell, thus functioning in vivo in a CD8 independent manner. The TCR α and β chains from the TIL were cloned and used to transduce peripheral blood lymphocytes. In vitro experiments using sorted CD4⁺ and CD8⁺ populations and antibody blockade of co-receptors demonstrated that this TCR was both CD4 and CD8 independent. In a HLA-A2/K^b transgenic mouse model, transfer of this anti-tyrosinase TCR into mouse splenocytes led to CD4 and CD8 independent antigen reactivity against melanoma cells in vitro. Adoptive transfer of TCR transduced T cells led to regression of established B16/A2-K^b

melanoma in lymphodepleted mice. Mice received either equal numbers of TCR transduced CD8+, CD4+ or a combination of CD8+ and CD4+ T cells. All groups demonstrated a statistically significant delay in tumour growth and a equal anti tumour response was seen in all 3 groups with no significant difference between the 3 groups. Chabra et al transduced CD4+ T cells with a TCR specific for the HLA-A2 restricted melanoma antigen, MART-1 (289). These CD4+ T cells demonstrated a Th1 cytokine profile in response to specific antigen and also had granule mediated cytotoxicity upon recognition of peptide loaded targets or melanoma cell lines expressing MART-1. CD4+ T cells were as efficient as CD8+ T cells transduced with the same TCR at direct cytotoxicity and were of the same functional avidity. This TCR was fully CD8 independent when expressed in CD4+ T cells.

In contrast, the F5-TCR is a CD8 dependent TCR and when expressed alone in CD4+ T cells can not provide tumour protection in the absence of CD8+ T cells. Previous work in our lab using F5-TCR CD4+ T cells has demonstrated that the F5-TCR CD4+ T cells could provide help for F5-TCR CD8+ T cells to eradicate EL4-NP tumour cells in vivo. A non tumour protective dose of F5-TCR CD4+ T cells could lead to tumour eradication if adoptively transferred in conjunction with F5-TCR CD8+ T cells. However the adoptive transfer of F5-TCR CD4+ T cells without functional F5-CD8+ T cells led to progressive tumour growth in all mice (4). These current experiments have gone on to explore whether class I restricted CD4+ T cells could eradicate tumours following adoptive transfer, even in the absence of tumour specific CD8+ T cells. F5-TCR + CD3 CD4+ T cells did initially control the tumour growth

more efficiently than F5-TCR CD4⁺ T cells. All mice that received adoptive transfer of F5-TCR CD4⁺ T cells died of progressive tumour growth, with tumour growth occurring at a similar rate to that seen when mock transduced CD4⁺ T cells were adoptively transferred. In contrast, none of the mice that received F5-TCR + CD3 CD4⁺ T cells died of progressive tumour growth. However the full extent of tumour protection provided by these cells could not be fully realized due to the sudden onset of toxicities in all mice that received F5-TCR + CD3 CD4⁺ T cells.

Following adoptive transfer in vivo into irradiated syngeneic mice who had received tumour challenge, F5-TCR + CD3 CD4⁺ T cells showed enhanced expansion in vivo compared to F5-TCR CD4⁺ T cells. There was a significant difference in expansion within the bone marrow, spleen and lymph nodes of the recipient mice. The increased surface expression of the transduced TCR leads to enhanced sensitivity to peptide and enhanced expansion in response to specific antigen in vivo. In addition, when analyzing later time points post adoptive transfer, there was a significantly higher proportion of adoptively transferred cells seen in the bone marrow and spleen of mice receiving TCR + CD3 CD4⁺ T cells suggesting that in addition to having superior initial expansion in vivo, these CD4⁺ T cells also persisted for longer and at higher levels than F5-TCR CD4⁺ T cells. Using in vivo imaging, it was clearly demonstrated that F5-TCR + CD3 CD4⁺ T cells trafficked to the tumour bed at a faster rate and in higher numbers than F5-TCR CD4⁺ T cells. These superior in vivo effects were due to the enhanced sensitivity to peptide arising from higher levels of F5-TCR expression when CD3 and F5-TCR are co-

transduced in CD4⁺ T cells. As a result of the enhanced expansion, persistence and faster trafficking to the tumour site, F5-TCR + CD3 CD4⁺ T cells are able to more effectively reduce tumour burden compared to F5-TCR CD4⁺ T cells. This superior anti-tumour effects is mediated even in the absence of antigen specific CD8⁺ T cells.

The in vivo experiments using bone marrow chimeric mice support the hypothesis that class I restricted CD4⁺ T cells can respond to antigen presented by non professional APCs. Whilst the results of the tumour protection are more difficult to interpret due to the toxicity seen, there is definite tumour protection seen following adoptive transfer of F5-TCR + CD3 CD4⁺ T cells into recipient mice of a differing bone marrow haplotype. In this experimental set up, the adoptively transferred cells can respond to antigen presented by the H2^b expressing tumour cells despite lack of professional APCs expressing H2^b class I molecules.

Whilst these experiments have shown that the in vivo function of class I restricted CD4⁺ T cells can be improved by the co-transduction of CD3. These CD4⁺ T cells can reduce the tumour burden compared to CD4⁺ T cells transduced with TCR alone. The exact mechanism by which the CD4⁺ T cells reduce tumour burden in vivo has not been explored and it is not clear whether this is due to direct cytotoxicity of the adoptively transferred cells or of a helper function of the class I restricted CD4⁺ T cells. Given that cytotoxic function can be provided by CD8⁺ T cells, it would be most effective if class I restricted CD4⁺ T cells were able to provide help in vivo and more

experiments are required to explore this in detail. Clearly however the additional tumour protection provided by the F5-TCR + CD3 CD4⁺ T cells is completely negated by the severe toxicity that is seen in the mice receiving the F5-TCR + CD3 CD4⁺ T cells. In the next chapter, experiments were carried out to try to explore in further detail the exact mechanism of these toxicities.

CHAPTER 5 – INVESTIGATION OF IN VIVO TOXICITY OF F5-TCR + CD3

CD4+ T CELLS

5.1 Introduction

Adoptive Transfer of F5-TCR + CD3 CD4+ T cells into sub lethally irradiated tumour challenged mice resulted in unexpectedly high levels of toxicity resulting in marked reduction in overall survival. These effects were seen only in mice receiving CD4+ T cells expressing F5-TCR and additional CD3 and not in mice receiving F5-TCR CD4+ T cells. Thus the toxicity that is seen may arise secondary to the increased expression of either the introduced TCR or the endogenous TCR. An alternative possibility is that the toxic effects are due to mispairing of the introduced and endogenous TCR leading to generation of TCR of unknown specificities.

5.2 Aims and Hypothesis

The aim of this chapter was to explore in more detail the mechanisms underlying the increased toxicity seen following the adoptive transfer of F5-TCR + CD3 CD4+ T cells. It was hypothesized that the higher expression level of the introduced TCR led to increased auto reactivity and targeting of normal tissues in the context of sub lethal irradiation. Experiments were designed to try to determine whether the toxic effects were due solely to the increased expression level of the introduced TCR or whether there was any

evidence for of significant levels of TCR mispairing or also any signs of toxicity being related to upregulation of the endogenous TCR.

5.3 Toxicity of F5-TCR CD3 CD4+ T cells is not related to dose of adoptively transferred population.

The toxicity that was observed in the mice receiving F5-TCR + CD3 CD4+ T cells may be due to targeting of normal tissue by the adoptively transferred CD4+ T cells. Alternatively, the marked in vivo expansion of F5-TCR + CD3 CD4+ T cells may generate massive cytokine release leading to bystander damage of normal host tissues via the release of pro inflammatory cytokines. It may therefore be possible to limit the toxicity by reducing the dose of antigen specific T cells adoptively transferred.

To explore this in more detail, a tumour challenge experiment was designed using 3 different doses of F5-TCR + CD3 CD4+ T cells: 1×10^6 , 0.3×10^6 and 0.03×10^6 F5-TCR + CD3 CD4+ T cells. The irradiation dose, tumour cell dose and timing of tumour inoculation were the same as in previous experiments.

Tumours grew progressively in all groups until day 6 post tumour challenge and then tumour growth plateaued in the 1×10^6 dose group. In the 0.3×10^6 dose group, tumour growth peaked at day 8 post transfer and then plateaued. In the 0.03×10^6 dose group tumour growth continued until day 18 post transfer and then plateaued. Control of tumour was most effective in mice

that received 1×10^6 F5-TCR + CD3 CD4+ T cells and as would be expected the mice receiving the lowest dose of T cells had the fastest rate of tumour progression although none had fatal tumour progression. The rate of tumour growth was still less than that seen in mice receiving mock-transduced CD4+ T cells or F5-TCR CD4+ T cells (Figure 32).

Mice that received 1×10^6 F5-TCR + CD3 positive cells had very rapid onset of cachexia and increase in clinical severity score resulting in sacrifice of all mice between days 10-11 post tumour challenge. Toxicity was also observed at the lower doses of 0.3×10^6 and 0.03×10^6 F5-TCR + CD3 CD4+ T cells with fatal cachexia developing by day 13-19 and day 19-23 post tumour challenge respectively. Onset of toxicity thus was delayed with a reduction in cell number transferred but it was still observed in all mice in the lowest cell dose tested. As expected, adoptive transfer of lower numbers of F5-TCR + CD3 CD4+ T cells led to less efficient control of tumour growth.

5.4 Toxicity is independent of cognate antigen.

Toxicity was observed in mice that received CD4+ T cells that had been co-transduced with F5-TCR and additional CD3 but did not occur in recipients of CD4+ T cells transduced with the F5-TCR alone. Initial experiments were performed in mice expressing the cognate peptide presented by class I MHC on EL4-NP tumour cells. However, it was not clear if the observed toxicity was dependent on antigen specific expansion of the F5-TCR + CD3 CD4+ T cells. In order to explore this, adoptive transfer experiments were carried out

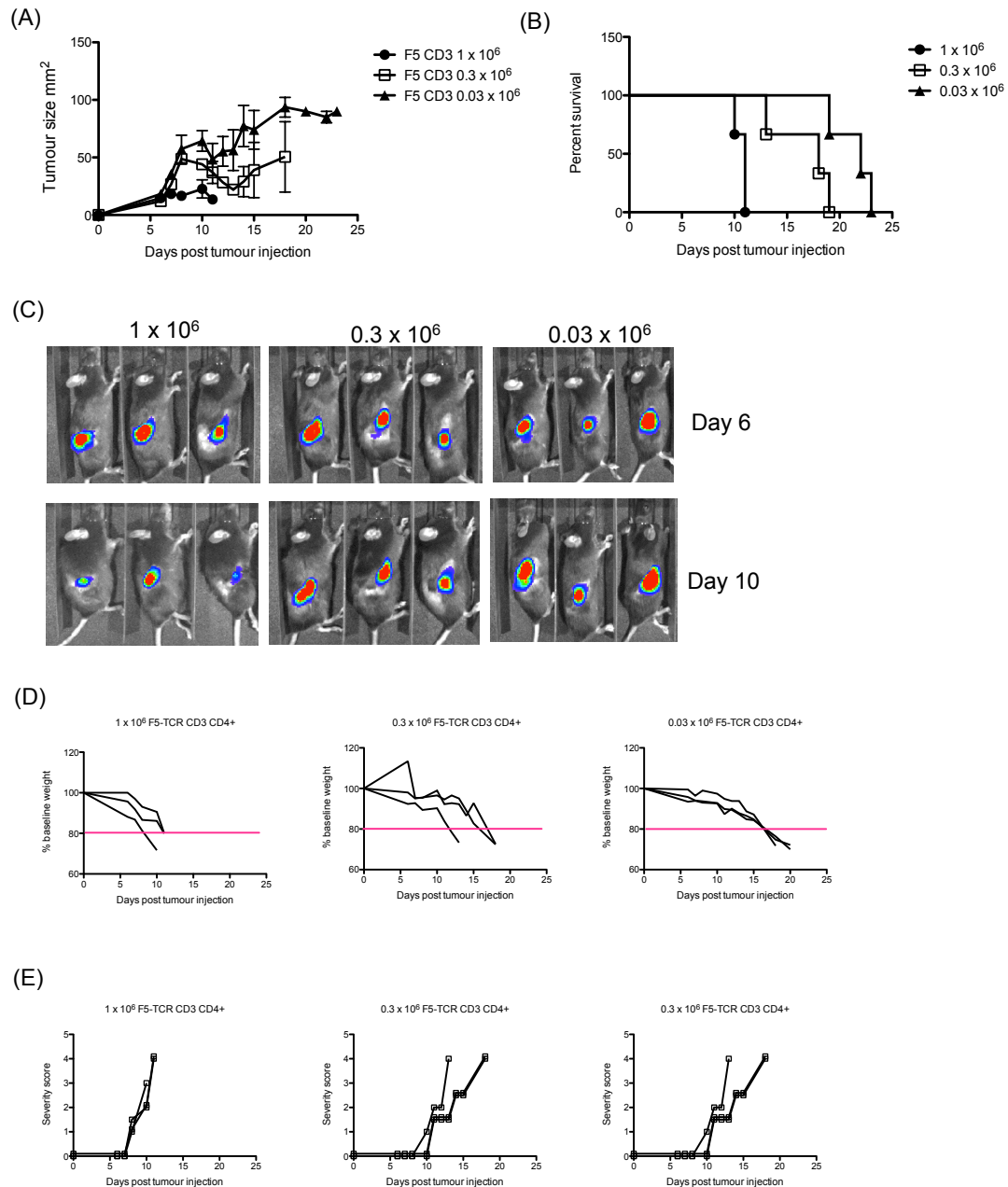


Figure 32 – Toxicity is delayed by reduction in dose of adoptively transferred F5-TCR CD3 + CD4+ T cells. C57BL/6 mice were sub lethally irradiated and then given a tumour challenge with 1 x 10⁶ EL4-NP tumour cells on Day 0. On Day 1, the following doses of F5-TCR CD3 + CD4+ T cells were administered: 1 x 10⁶, 0.3 x 10⁶ or 0.03 x 10⁶. (A) Mean tumour area measured by calipers. (B) Kaplan Meier survival curves post tumour challenge. (C) Bioluminescent signal at site of tumour growth between day 6 and day 10 post tumor challenge (D) % weight loss from baseline of individual recipient mice. (D) Severity score post tumour challenge in individual mice. Results shown are based on 1 experiment with n=3 mice/group.

where one group of mice was not challenged with tumour. Recipient mice were irradiated with 5.5 Gy irradiation on day 0 and one group were challenged with 1×10^6 EL4-NP tumour cells subcutaneously and one group received no tumour challenge. Adoptive T cell transfer was carried out on day 1 with 1×10^6 F5-TCR + CD3 CD4+ T cells per group. Mice were then monitored for weight loss and clinical severity scores as in previous experiments.

The results shown are from 4 independent experiments (F5-TCR + CD3 CD4+ no antigen n=15, F5-TCR + CD3 CD4+ n=12). In the mice not challenged with tumour, 13 out of 15 mice had to be sacrificed secondary to marked cachexia and/or high severity scores. This occurred between 10 and 19 days post irradiation. No significant difference in survival was observed between the groups regardless of exposure to antigen (tumour cells) ($p=0.68$). In the absence of antigen, the median survival was 17 days and in the presence of antigen, the median survival was 12 days (Figure 33A). Plots of maximal weight loss demonstrated that the extent of weight loss was not significantly different with or without antigen although the mean severity score was higher in the presence of cognate antigen (Figure 33B and 33C). Thus toxicity observed post adoptive transfer of F5-TCR + CD3 CD4+ T cells was not significantly different whether mice received challenge with tumour expressing cognate antigen or not.

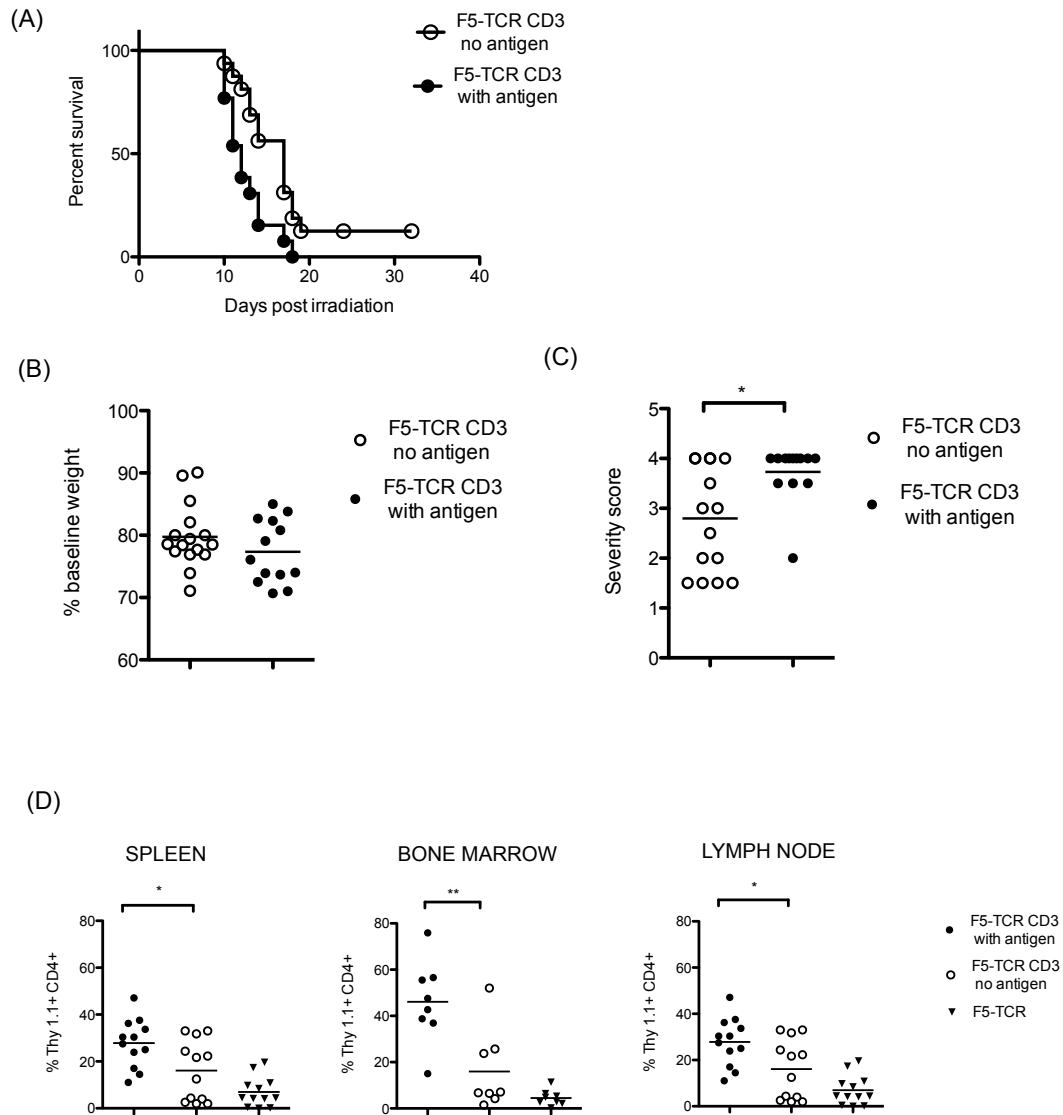


Figure 33 – Toxicity arising following adoptive transfer of F5-TCR + CD3 CD4+ T cells is not dependent on presence of cognate antigen. Mice were irradiated with 5.5 Gy on day 0; one group were challenged with 1×10^6 EL4-NP tumour cells subcutaneously and one group did not receive tumour challenge. Adoptive Transfer of 1×10^6 F5-TCR CD3 CD4+ T cells was carried out on day 1. (A) Kaplan Meier survival curves post adoptive transfer (B) Maximal weight loss (C) Maximal severity score (D) Comparison of % Thy 1.1 CD4+ T cells in F5-TCR CD3 (No antigen), F5-TCR CD3 (with antigen). Data from experiments using F5-TCR only CD4+ T cells is shown for comparison. Results shown are from 4 independent experiments, F5-TCR + CD3 CD4+ no antigen n= 15; F5-TCR + CD3 CD4+ with antigen n=12).

In addition, samples from spleen, bone marrow and lymph node were analyzed on the day of sacrifice as in previous experiments to look at persistence or expansion of the adoptively transferred T cells. From the F5-TCR + CD3 no antigen group, spleen, bone marrow and lymph node samples were only available from 11, 6 and 7 mice respectively. Within the spleen, the mean % of Thy 1.1+ CD4+ T cells was 16.1% (range 2-33%) in the no antigen group and 27.8% (range 11-47%) in the group that was antigen challenged ($p=0.04$). In the bone marrow the mean % of Thy1.1+ CD4+ T cells was 15.9% (range 1.5-52%) in the no antigen group and 46.1% (range 15-75.9%) in group that received antigen challenge ($p=0.007$). Within the lymph node the mean % of Thy1.1+ CD4+ T cells was 9.8% (range 1.4-24.8%) in the no antigen group compared to 38.7% (range 24.9-51.7%) in the group that received antigen ($p=0.0002$) (Figure 33D). Thus the mean % of Thy 1.1+ CD4+ T cells was significantly lower in the absence of antigen following the adoptive transfer of F5-TCR CD3 CD4+ T cells compared to in the presence of cognate antigen. This would suggest that the toxicity seen is not directly due to expansion of F5-TCR CD3 CD4+ T cells driven by recognition of NP peptide-MHC. However it is possible that the α and β chains of the F5 TCR are mispairing and that the adoptively transferred T cells expressing mispaired TCRs expand following recognition of an unknown self antigen. It would be expected that only a proportion of the adoptively transferred T cells will express a mispaired TCR, with the majority expressing the F5 TCR. Therefore in the presence of cognate NP peptide, expansion of the adoptively transferred F5-TCR expressing CD4+ T cells may be enhanced.

5.5 Toxicity occurs following adoptive transfer of CD4⁺ T cells that express additional CD3 in the absence of transduced TCR or mispaired TCR.

To determine whether toxicity was related in part or in whole due to the up regulation of endogenous TCR, CD4⁺ T cells were transduced with additional CD3 only. Toxicities observed in these mice would be subsequent to the up regulation of the endogenous TCR in transferred T cells or secondary to a toxic effect of the additional CD3 molecule itself. The experimental set up was as in the previous experiment: Recipient mice received sub lethal irradiation with 5.5 Gy on day 0 and then on day 1 underwent adoptive transfer of 1×10^6 CD3 only CD4⁺ T cells (n=12) or of 1×10^6 F5-TCR + CD3 CD4⁺ T cells (n=13) or of 1×10^6 mock transduced CD4⁺ T cells (n=5). No tumour challenge was administered in this experiment. Mice were monitored by weights and clinical severity scores as in previous experiments. Results shown are from 3 independent experiments.

In the CD3 CD4⁺ T cells group, 6 of the 12 mice were sacrificed either secondary to weight loss or increases in clinical severity score. The remaining 6 mice remained well with no toxicity observed until the end of the experiment. In the F5-TCR + CD3 CD4⁺ group, 11 out of 13 mice were sacrificed as a result of increased weight loss and/or high clinical severity scores. All 5 mice in the mock group remained well throughout the experiment with no weight loss or increase in clinical severity score observed. When compared to the survival of mice that received F5-TCR + CD3 CD4⁺ T cells (and no tumour

challenge), the survival of the CD3 only CD4+ group was superior. The median survival of CD3 CD4+ group was 26 days compared to 15 days in the F5-TCR + CD3 CD4+ group ($p=0.006$) (Figure 34A).

Comparison of maximal weight loss and maximal severity score between the groups did not demonstrate any significant increase in weight loss or clinical severity scores when comparing the CD3 CD4+ T cell group and F5-TCR + CD3 CD4+ T cell groups. Compared to the mice that received mock transduced CD4+ T cells both the CD3 CD4+ T cell group and the F5-TCR + CD3 CD4+ T cell group lost significantly higher amounts of weight from baseline and had significantly higher clinical severity scores. Whilst toxicity was less in the CD3 only group compared to the F5-TCR + CD3 group, half of the mice showed evidence of weight loss and high clinical severity scores and thus had a reduction in overall survival. It is hypothesized that the reduced survival that is seen in the CD3 only group is mediated by upregulation of the endogenous TCR generating autoreactive T cells which target normal tissues leading to GVHD (Figure 34B and 34C).

There was no significant difference in the mean percentage of Thy 1.1+ CD4+ T cells seen within the bone marrow or lymph node in the CD3 only group or the F5-TCR + CD3 group (Figure 34D). Within the spleen, a significantly higher percentage of Thy 1.1+ CD4+ T cells was seen in the F5-TCR + CD3 group compared the CD3 only group. Not surprisingly, mice that received F5-TCR + CD3 CD4+ T cells and tumour challenge had a significantly higher %

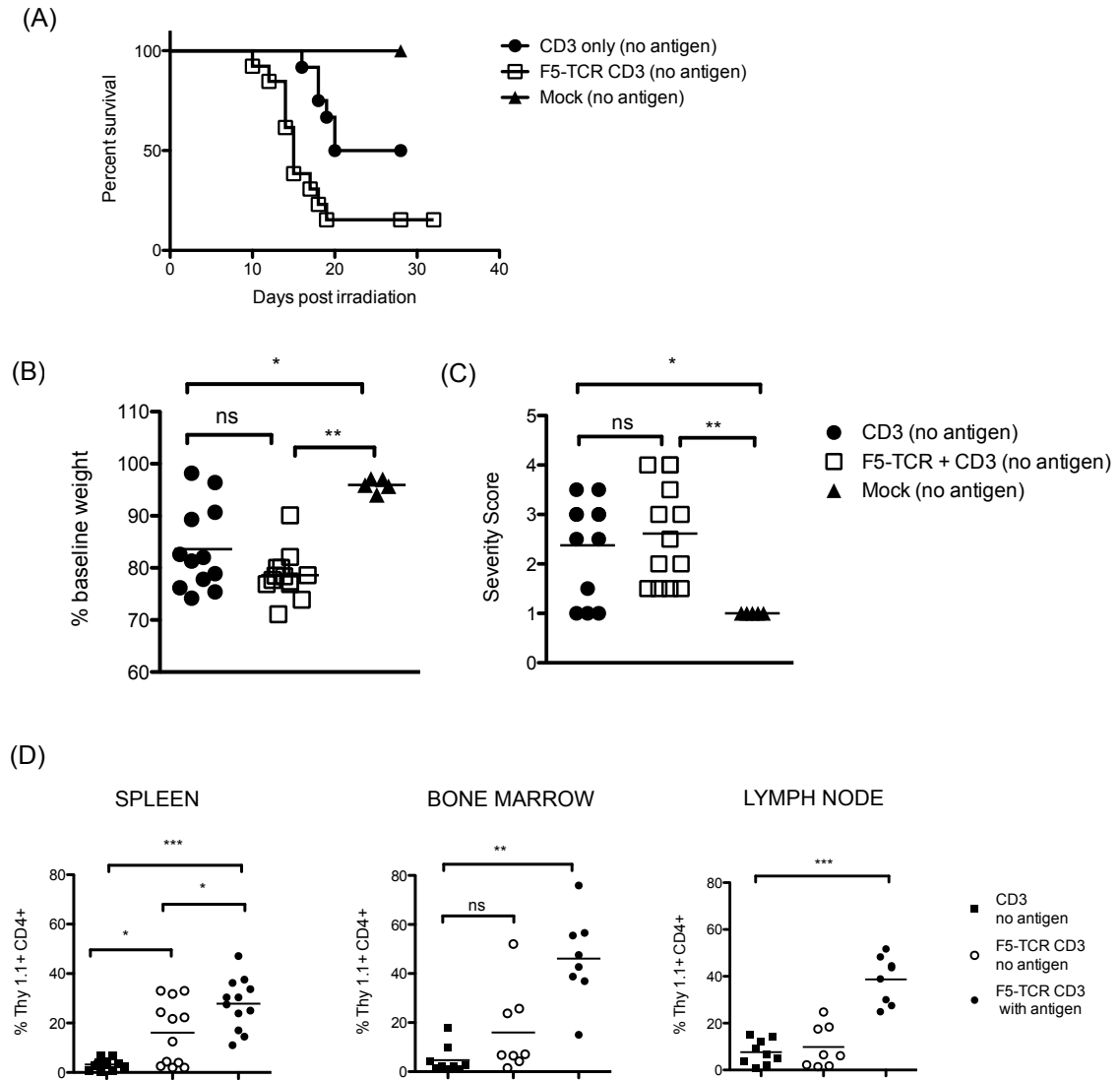


Figure 34 – Toxicity arises following adoptive transfer of CD4+ T cells transduced with additional CD3 but without introduction of the F5 TCR. (A) Kaplan Meier Plots of Overall Survival following adoptive transfer of CD3 only CD4+T cells and F5-TCR CD3 CD4+ T cells. (B) Maximal Weight loss (C) Mean Severity Score (D) Mean % Thy 1.1+ CD4+ T cells in spleen, bone marrow and lymph node following adoptive transfer (results from experiments using F5-TCR + CD3 CD4+ T cells challenged with tumour are shown for comparison). Results shown are from 3 independent experiments, CD3 CD4+ n=12, F5-TCR + CD3 CD4+ n=13, Mock n=5).

of adoptively transferred cells present in all organs analyzed compared to mice receiving CD3 only CD4⁺ T cells in the absence of antigen.

5.6 Reducing the irradiation dose prior to adoptive transfer of F5-TCR CD3 CD4⁺ T cells prevents toxicity

Dose-dependent lymphodepletion is well documented for radiation therapy. Radiation-induced reduction of host haematopoiesis increases the relative availability of homeostatic cytokines such as IL-7 and IL-15 for the adoptively transferred T cells, promoting their proliferation *in vivo*. However, higher irradiation doses may cause additional tissue damage and inflammation in the recipient. Damaged host tissues secrete pro-inflammatory cytokines such as TNF- α and IL-1 together with other danger signals leading to activation of host APCs. Furthermore, radiation-induced damage to the gastrointestinal tract results in the systemic translocation of LPS through the bowel wall further activating APCs (228) (229). Donor T cells which are recruited to the inflamed tissues are then activated by the APCs resulting in proliferation and differentiation. Further production of inflammatory cytokines by activated donor T cells amplifies the harmful pro-inflammatory response leading to further tissue destruction. There is a linear relationship between radiation dose, tissue damage, cytokine storm and acute GVHD related mortality (230) (231) (232). It is therefore expected that high doses of irradiation may have exacerbated potential harmful T cell mediated toxicities arising post adoptive transfer of F5-TCR + CD3 T cells.

Experiments were performed to determine whether the toxicity mediated following adoptive transfer of F5-TCR + CD3 CD4⁺ T cells could be reduced or prevented by reducing the dose of conditioning irradiation. In these experiments, recipient mice received 4Gy irradiation on day 0 and then 4 hours post irradiation were injected with 1×10^6 EL4-NP luciferase positive cells subcutaneously. On day 1, mice were injected intravenously with 1×10^6 F5-TCR + CD3 CD4⁺, F5-TCR only CD4⁺ or Mock transduced CD4⁺ T cells. There were 5 mice per group. In the previous experiments all mice received 5.5 Gy.

In the F5-TCR + CD3 group, there was more efficient control of tumour growth than in the mice that received F5-TCR only CD4⁺ T cells or mock-transduced CD4⁺ T cells. The mean tumour area was lower at all time points measured in mice receiving F5-TCR + CD3 CD4⁺ T cells. No mice in the F5-TCR + CD3 group had progressive tumour growth. The F5-TCR only group had the fastest rate of tumour growth and the highest tumour burden, which peaked at day 12 post injection and then regression was observed in 3/5 mice. In the mock-transduced group the tumour growth peaked at day 15 post injection and then regressed in 3/5 mice (Figure 35).

In addition to reduced homeostatic expansion potential for transferred T cells after lower doses of radiation, there will be greater numbers of residual host T cells which may play a role in tumour rejection. Host T cells will be able to respond to NP peptide expressed by the EL-4 NP lymphoma cells and this will

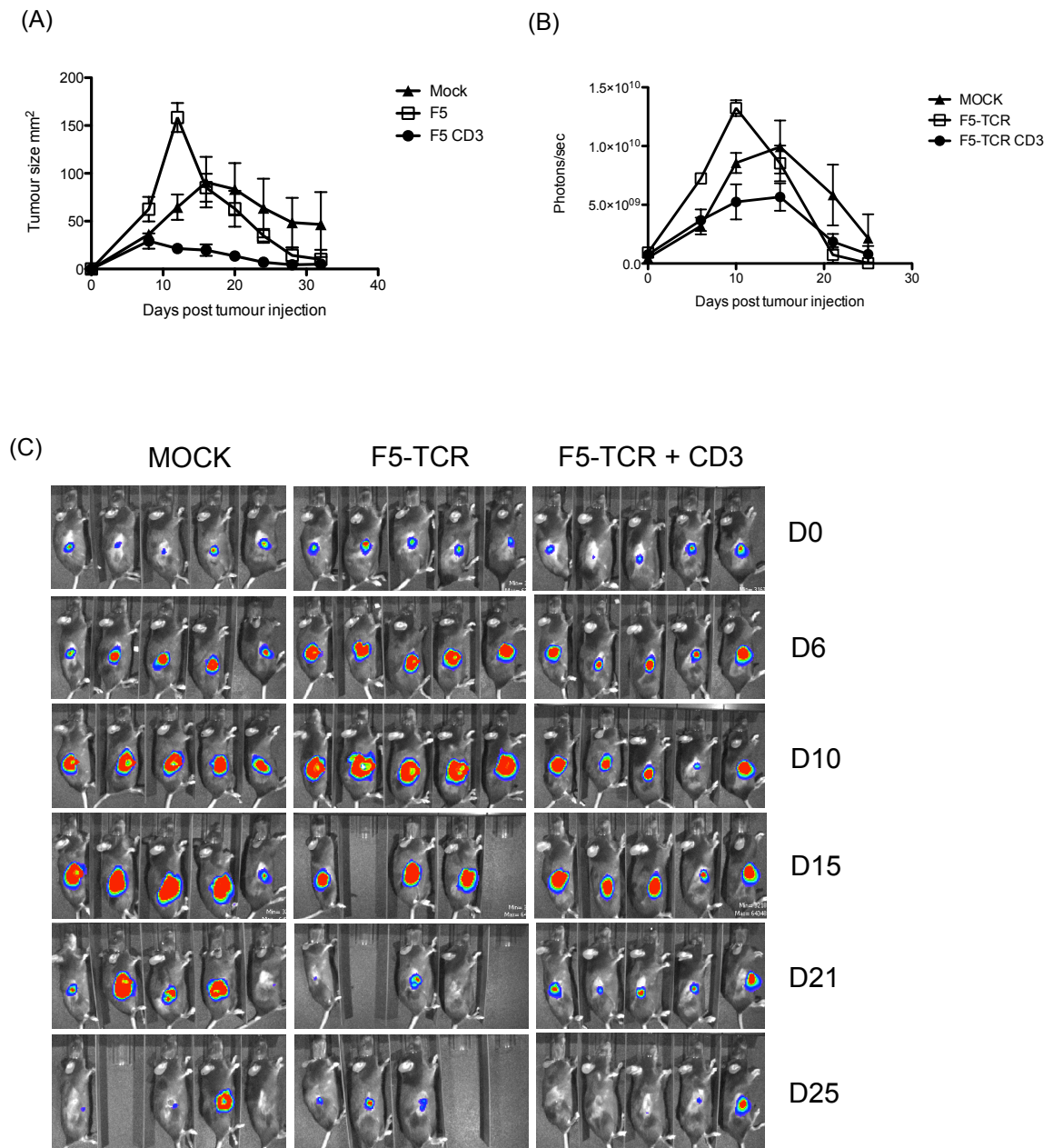


Figure 35 – Reduction in irradiation dose leads to a reduction in toxicity. C57BL/6 recipient mice were irradiated with 4 Gy on day 0 and injected with 1×10^6 EL4-NP luciferase+ tumour cells. On day 1 mice were injected I.V with 1×10^6 F5-TCR + CD3 CD4+ T cells, F5-TCR CD4+ T cells or mock transduced CD4+ T cells and followed for tumour growth, weight loss and clinical severity score. (A) Tumour Area measured by calipers. (B) Mean bioluminescent signal in photons/second recorded at site of tumour growth. (C) Bioluminescent signal at site of tumour growth between day 0 and day 25 post tumour challenge. Results shown are from one experiment with n=5 mice/group.

contribute to tumour rejection, supported by the efficient tumour rejection in mice that received mock-transduced CD4⁺ T cells.

Weight was well maintained in all mice observed and no increases in severity scores were documented (Figure 36A). There was no significant difference in survival between any of the 3 groups (Figure 36B). Mice were monitored until day 40 post tumour injection and then culled for analysis of T cell persistence. These results were confirmed in a second independent experiment with a further 3 mice per group.

After 4 Gy conditioning, the % of Thy 1.1⁺ CD4⁺ T cells detectable in the spleen, bone marrow and lymph node was significantly higher in mice that had received F5-TCR + CD3 CD4⁺ T cells. In mice that received F5-TCR + CD3 CD4⁺ T cells the mean percentage of Thy 1.1⁺ CD4⁺ T cells in the bone marrow, spleen and lymph node was 3.4%(1-2%-7.1%), 3.5%(0.9%-5.4%) and 2.4%(0.9%-4.8%) respectively. In the mice that received TCR only CD4⁺ T cells the mean percentage of Thy 1.1⁺ CD4⁺ T cells within the bone marrow, spleen and lymph node was 0.4% (0.1-0.8%) (p=0.004), 1.0% (0.2%-2%) (p=0.002) and 1.1%(0.2-2.5%) (p=0.01) (Figure 36D).

In mice conditioned with 5.5Gy, there were significantly more adoptively transferred cells detectable within all organs i.e. higher percentage of Thy 1.1⁺ CD4⁺ T cells (Figure 36E). The analysis was performed at a later time point in the experiments using 4Gy irradiation (Day 40 post tumour injection

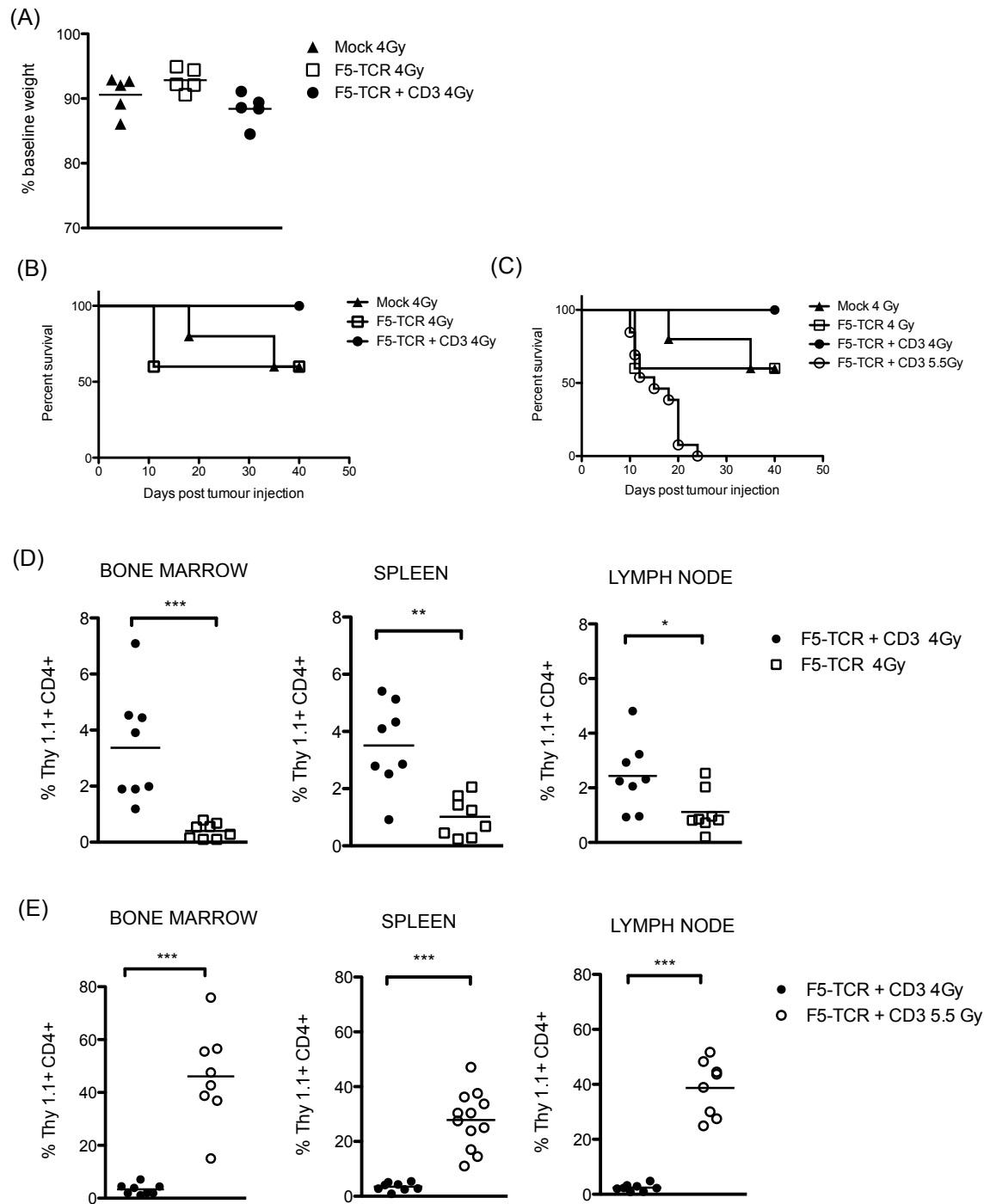


Figure 36 – Reduced irradiation dose leads to improved overall survival that is associated with a reduction in expansion of adoptively transferred cells. Experimental set up was as in figure ?. (A) Mean % of baseline weight (B) Kaplan Meier Plot of overall survival of mice irradiated with 4Gy (C) Kaplan Meier Plot of overall survival comparing mice irradiated with 4Gy and 5.5Gy (D) % Thy 1.1+ CD4+ T cells in Bone Marrow, Spleen and Lymph node of F5-TCR and F5-TCR + CD3 groups. (E) % Thy 1.1+ CD4+ T cells in bone marrow, spleen and lymph node comparing mice that received F5-TCR + CD3 CD4+ T cells irradiated with 4Gy versus 5.5 Gy.

as compared to day 10-25 post tumour injection) as the mice had significantly longer survival times.

The overall survival of mice that received F5-TCR + CD3 CD4⁺ T cells following 4Gy of irradiation was superior to those that received the same dose of F5-TCR + CD3 CD4⁺ T cells but 5.5 Gray of irradiation (p=0.002) (Figure 36C).

5.7 TCR + CD3 co-transduced CD4⁺ T cells confer more toxicity in vivo than equivalent numbers of TCR + CD3 co-transduced CD8⁺ T cells

The following experiments were performed to compare the in vivo toxicity following the adoptive transfer of F5-TCR + CD3 CD8⁺ T cells and F5-TCR + CD3 CD4⁺ T cells. Experiments previously performed in our laboratory with CD8⁺ T cells co-transduced with F5-TCR and CD3 had not demonstrated toxicity in the same EL4-NP tumour model.

C57BL/6 recipients were irradiated with 5.5 Gy irradiation and then 4 hours later tumour challenge was administered subcutaneously with 1×10^6 EL4-NP cells as previously described. On day 1, mice were injected intravenously with 1×10^6 F5-TCR only CD8⁺, 1×10^6 F5-TCR + CD3 CD8⁺ or 1×10^6 F5-TCR + CD3 CD4⁺ T cells. Mice were then monitored for tumour burden and for weight loss and development of high clinical severity scores. The results shown are from 2 independent experiments (F5-TCR CD3 CD8⁺ n=10; F5-TCR CD3 CD4⁺ n=10; F5-TCR only CD8⁺ n=5).

Mice that received F5-TCR + CD3 CD4⁺ T cells died secondary to toxicity with a median survival of 13.5 days post tumour injection. In the F5-TCR + CD3 CD8⁺ T cell group, 8/10 mice died secondary to toxicity with a median survival of 15.5 days post tumour challenge. In the F5-TCR only CD8⁺ T cell group, 1/5 mice died secondary to toxicity. The remaining 4 mice survived with no toxicity (i.e. no cachexia or increased clinical severity scores). The F5-TCR only CD8⁺ group had a superior survival compared to both F5-TCR + CD3 CD8 group ($p=0.048$, log rank) and to the F5-TCR + CD3 CD4⁺ group ($p=0.003$, log rank test). No progressive tumour growth was observed in any of the mice (Figure 37A, B and C).

It was observed that mice that receiving F5-TCR + CD3 CD4⁺ T cells had increased numbers of Thy 1.1⁺ CD4⁺ T cells within the bone marrow, spleen and lymph node compared to the F5-TCR + CD3 CD8⁺ T cell group and the F5-TCR only CD8⁺ T cell group. As the mice that received tumour specific CD8⁺ T cells developed minimal tumour growth compared to F5-TCR + CD3 CD4⁺ T cell group, it may have been that all the tumour specific CD8⁺ T cells were retained within the tumour microenvironment (site of antigen) and not in the periphery.

Within the bone marrow, 51.2% (range 36.9-75.9) of viable lymphocytes were Thy 1.1⁺ CD4⁺ T cells in the F5-TCR + CD3 CD4⁺ group compared to 5.1% (2.2 -12.2) in the F5-TCR + CD3 CD8⁺ group (<0.0001). In the lymph node the percentage of Thy 1.1⁺ CD4⁺ T cells present was 29% (24.9%-51.7%) in

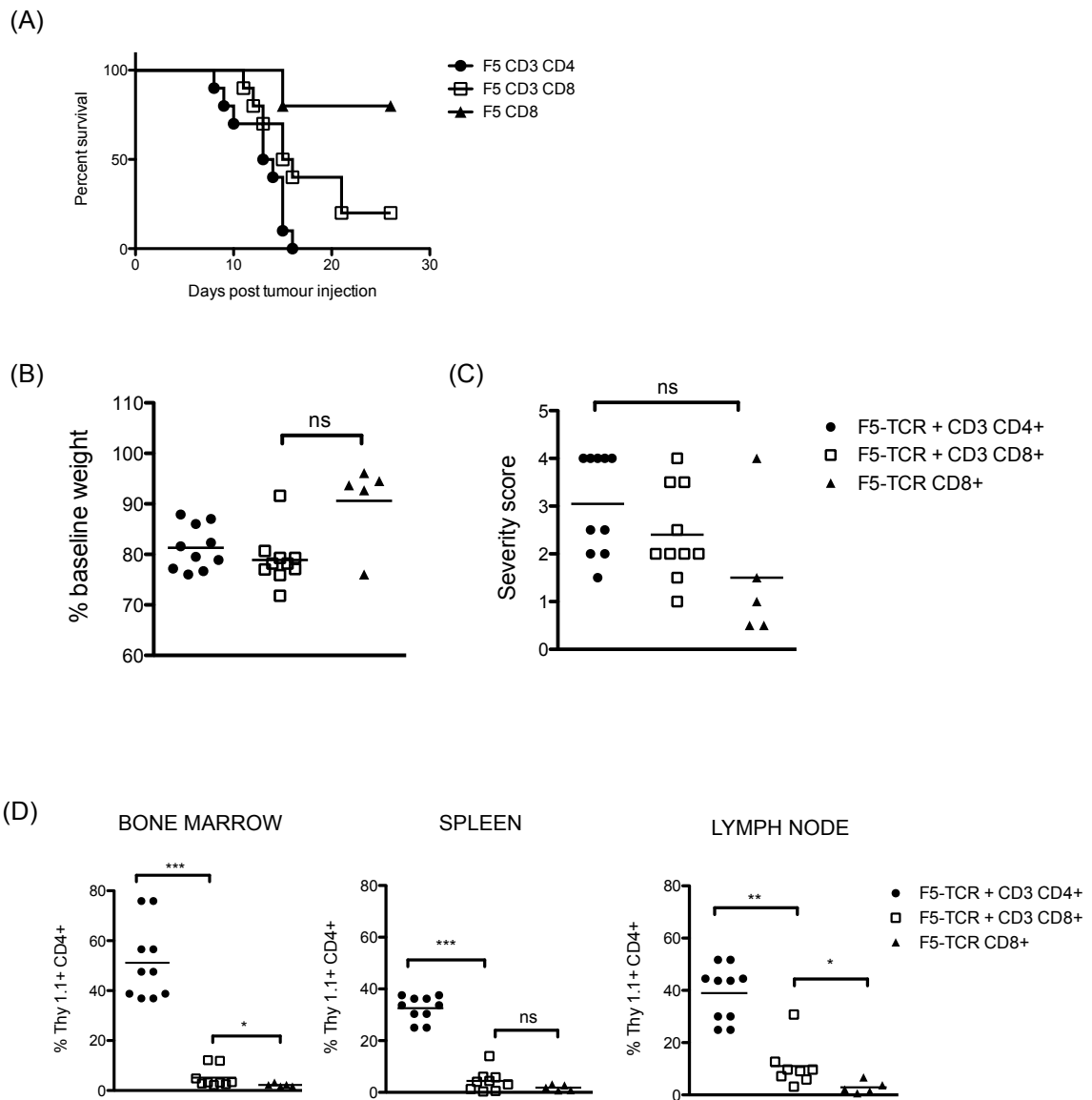


Figure 37 – Toxicity is demonstrated following adoptive transfer of F5-TCR + CD3 CD8+ T cells but is not as severe as that seen post adoptive transfer of F5-TCR + CD3 CD4+ T cells. Mice were irradiated with 5.5 Gy on day 0 and 4 hours post irradiation were injected subcutaneously with 1×10^6 EL4-NP tumour cells. On day 1 mice were injected I.V with 1×10^6 F5-TCR + CD3 CD4+, F5-TCR + CD3 CD8+ or F5-TCR CD8+ T cells. (A) Kaplan Meier Plot of overall survival post tumour challenge. (B) Maximal weight loss (C) Maximal severity score (D) % Thy 1.1+ CD4+ T cells in bone marrow, spleen and lymph node. Results shown are representative of 2 independent experiments. F5-TCR + CD3 CD4+ n=10, F5-TCR + CD3 CD8+ n=10, F5-TCR CD8+ n=5.

mice that received F5-TCR + CD3 CD4+ T cells compared to 11% (3.1%-30.8%) in mice that received F5-TCR + CD3 CD8+ T cells ($p=0.002$). In the spleen, the mean percentage of Thy 1.1+CD4+ T cells was 32.8% (25-37.6%) F5-TCR + CD3 CD4+ group compared to 4.4% (0.4%-14%) in the F5-TCR + CD3 CD8+ group ($p<0.0001$). Mice that received F5-TCR CD8+ T cells had the lowest percentage of Thy 1.1+ CD4+ T cells within all organs (bone marrow 2.2%(1.7-3.2); lymph node 2.9%(0.6%-6.7%); spleen 1.8%(0.8-3.1%) (Figure 37D).

Previous work in our lab using F5 TCR + CD3 CD8+ T cells has not demonstrated increased toxicity following adoptive transfer in the EL4-NP tumour challenge model compared to F5-TCR only CD8+ T cells (5). However in these studies a 3 fold lower dose of TCR modified T cells was transferred and TCR transduced T cells had been FACS sorted prior to adoptive transfer. CD8+ T cells had also been activated prior to transduction using IL-7 and Con-A rather than CD3/CD28 activation beads. All of these factors may have led to a reduction in toxicity from what was seen in the experiments reported here.

5.8 Enhanced TCR mispairing in the presence of additional CD3 may contribute to toxicity.

Bendle et al demonstrated evidence of TCR mispairing in a model in which OT-1 TCR transduced bulk T cells were adoptively transferred into irradiated mice. Post transfer, high dose IL-2 was administered to promote T cell

expansion. They found that on day 14 post transfer mice developed marked and rapidly progressive cachexia that was not seen in the recipients of untransduced T cells and this was shown to be secondary to generation of mispaired TCRs. They also did a small comparison of adoptive transfer of OT1 CD4⁺ T cells with OT-1 CD8⁺ T cells: both groups of mice developed toxicity but toxicity was much higher in the group that received CD4⁺ T cells compared to CD8⁺ T cells (157).

To explore whether the toxicity that was seen following adoptive transfer of F5-TCR + CD3 CD4⁺ T cells was due in part to TCR mispairing, a vector encoding only the β chain of the F5-TCR was generated (pMP71-F5 β) (Production of the vector is described in Chapter 2, Section 2.7).

CD4⁺ T cells were transduced with the F5-TCR + CD3 or with F5-TCR β + CD3 as per usual transduction protocols. Upregulation of the F5-TCR β chain expression was seen in the presence of additional CD3. However, unlike following transduction of the F5-TCR + CD3, no binding to specific pentamer was observed (Figure 38). Interestingly, the percentage of V β 11⁺ CD3⁺ cells were higher in the F5 β + CD3 transduced CD4⁺ T cells compared to the F5-TCR + CD3 CD4⁺ T cells.

To explore whether CD4⁺ T cells transduced with only the F5 β chain could mediate off target effects in vivo, the following experiment was carried out. C57BL/6 mice were irradiated with 5.5 Gray on day 0. No tumour challenge was administered. On day 1, 1×10^6 Mock CD4⁺, F5-TCR β CD4⁺ or F5-

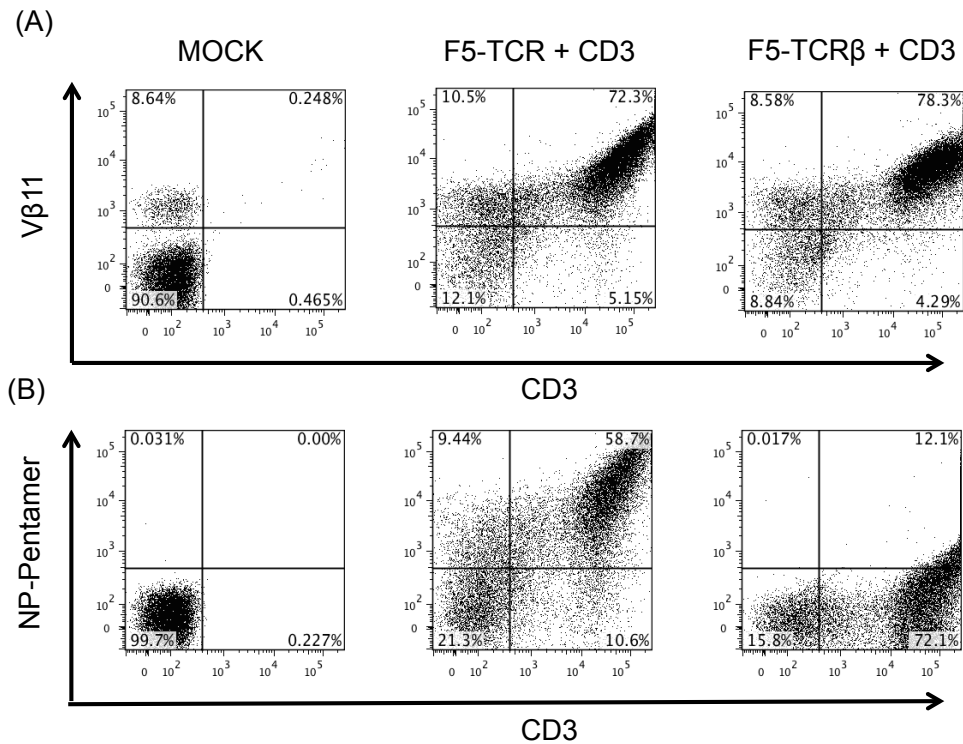


Figure 38 – Co-transduction of F5 β + CD3 leads to increased surface expression of the F5 β chain. CD4⁺ T cells were mock transduced or transduced with F5-TCR + CD3 or F5-TCR β + CD3 using standard transduction protocol. Comparison of staining of mock transduced, F5-TCR + CD3 and F5-TCR β + CD3 cells stained for expression of (A) V β 11 and (B) NP-Pentamer. Cells were initially gated on viable lymphocytes and then CD4⁺ T cells.

TCR β + CD3 CD4⁺ or CD3 only CD4⁺ T cells were adoptively transferred to the recipient mice. There were 5 mice per group (except for CD3 only, n=4). Mice were monitored for weight loss and by measurement of clinical severity score as all previous in vivo experiments.

In the F5-TCR β + CD3 group there was rapid development of cachexia and increased clinical severity scores in all 5 mice. Median survival in this group was only 10 days. In the F5-TCR β only group, all 5 mice also died of toxicity although the onset of weight loss and increase severity score was delayed compared to mice receiving F5-TCR β + CD3 CD4⁺ T cells. The median survival in F5-TCR β only group was 16 days. In the CD3⁺ only group, 1/4 mice developed cachexia and weight loss and was sacrificed 18 days post irradiation. None of the mice in the mock CD4⁺ T cell group developed any evidence of toxicity. Median survival time was not reached in the CD3⁺ only CD4⁺ T cell group or mock CD4⁺ T cell treated group and the experiment was finished on day 25 post irradiation (Figure 39).

The very rapid onset of toxicity and marked reduction in survival of mice receiving CD4⁺ T cells transduced with the F5-TCR β chain supports the hypothesis that the toxicity is being mediated (in whole or in part) by mispairing of the introduced TCR α or β chains with the endogenous α and β chains. In these experiments, the introduced F5-TCR β chain was able to pair with endogenous α TCR chains as the total number of V β 11⁺ cells was similar between the groups. These mispaired TCRs are of novel and unknown specificity and will not have been screened for auto reactivity by

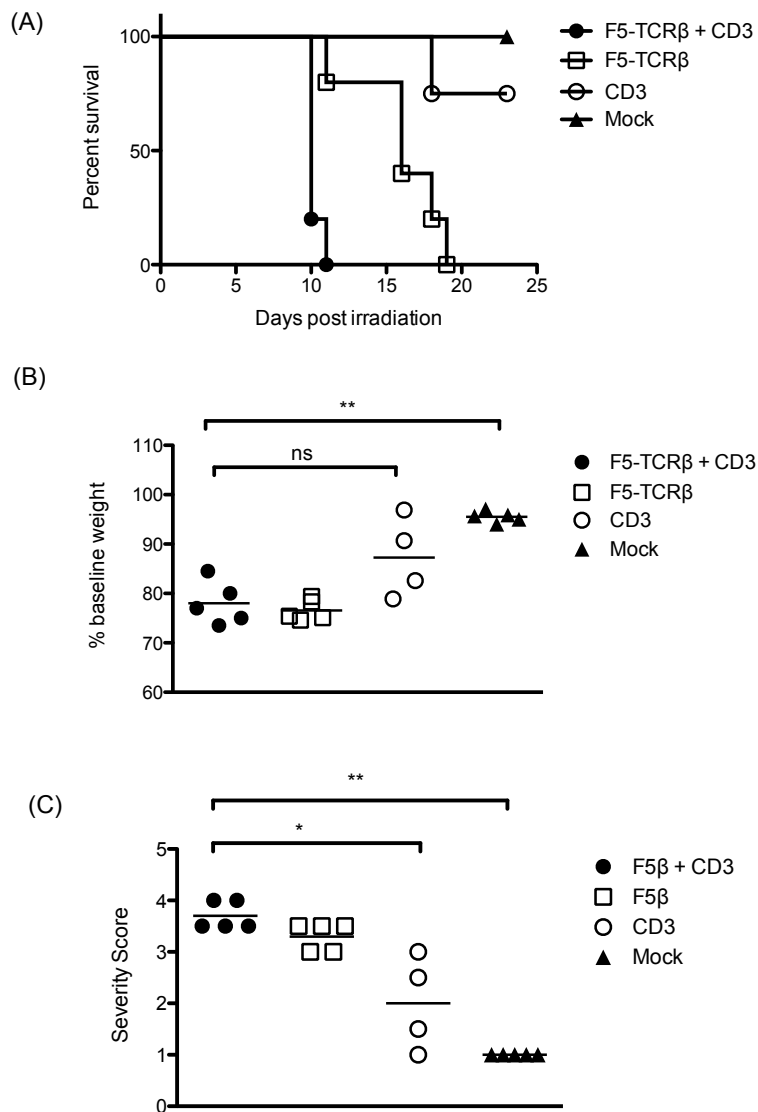


Figure 39 – Adoptive Transfer of CD4⁺ T cells transduced with F5 β chain leads to toxicity which is enhanced in the presence of additional CD3. C57BL/6 mice were irradiated with 5.5 Gy on Day 0, no mice received tumour challenge. On day 1, 1×10^6 Mock CD4⁺, F5-TCR β CD4⁺, F5-TCR β + CD3 CD4⁺ or CD3 only CD4⁺ T cells were adoptively transferred. (A) Kaplan Meier Plot of overall survival (B) Mean % baseline weight (C) Mean severity score (results shown are from 1 experiment, n=5/group (except CD3 only n=4)).

central tolerance processes. The off target effects of the mispaired F5-TCR β chain is greatly enhanced when CD3 is co-transduced (Figure 39). A greater expansion of the transferred Thy 1.1⁺ CD4⁺ T cells was observed in mice that received F5-TCR β + CD3 CD4⁺ T cells or F5-TCR β CD4⁺ T cells compared to mice receiving mock transduced or CD4⁺ T cells transduced with CD3 only (Figure 40). There was greater expansion of the adoptively transferred cells in the bone marrow, spleen and lymph nodes in the F5-TCR β + CD3 group and the F5-TCR β group. This data supports the hypothesis that the F5-TCR β chain mispairs with endogenous α chains. Expansion of the adoptively transferred cells may be promoted by binding of novel mispaired TCRs to self-antigen in vivo.

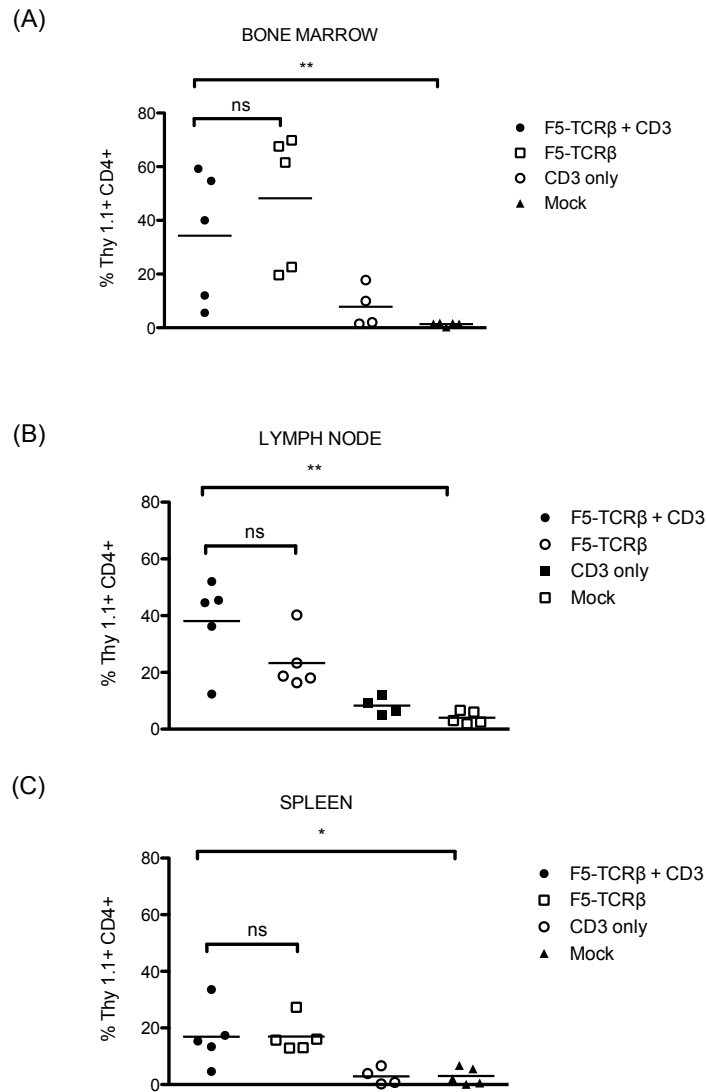


Figure 40 – F5-TCRβ + CD3 CD4+ T cells and F5-TCRβ CD4+ T cells have greater expansion post adoptive transfer than CD4+ T cells that lack transduced TCR α or β chains. Plots show mean % Thy 1.1+ CD4+ T cells in (A) Bone Marrow (B) Lymph Node (C) Spleen. (results shown are from 1 experiment, n=5/group except for CD3 only n=4). Analysis was carried out on day 10-25 post irradiation).

5.9 Discussion

Clinical trials utilizing the adoptive transfer of tumour specific gene modified T cells to treat malignancies have demonstrable clinical responses but there is increasing recognition of the on and off target effects arising in patients treated with TCR transduced T cells. On target refers to toxicity arising secondary to the effects of recognition of the target antigen in vivo whilst off target toxicities result from targeting of different self antigens which are not the cognate peptide for which the TCR is specific for. Off target toxicity may arise due to generation of novel TCR specificities by mispairing of the introduced TCR and the endogenous TCR but may also arise secondary to cross reactivity of the introduced TCR for self antigens other than the original target antigen.

These experiments had initially been designed to improve the function of class I restricted CD4⁺ T cells in tumour protection models although they revealed marked and unexpected toxicities following adoptive transfer into irradiated syngeneic recipients. Mice receiving F5-TCR + CD3 CD4⁺ T cells developed very rapid onset of cachexia and high clinical severity scores resulting in the premature death of all recipients of F5-TCR + CD3 CD4⁺ T cells. None of the mice that received F5-TCR CD4⁺ T cells or mock transduced CD4⁺ T cells developed this same pattern of weight loss with high clinical severity scores. However, potential toxic effects of the F5-TCR CD4⁺ T cells may have been masked in the tumour model as their survival was reduced due to progressive tumour growth. Alternatively, the functional

avidity of the F5-TCR CD4⁺ T cells may have been too low to mediate autoreactivity against self antigens. Thus it would appear that at least in part these toxicities were arising as a result of the high expression level of the introduced TCR or some other affect of the introduction of an excess of CD3 molecules. Further adoptive transfer experiments demonstrated that the toxicity was not dependent on T cell dose and still occurred following adoptive transfer of only 0.01×10^6 TCR⁺ CD3⁺ CD4⁺ T cells. So whilst reducing the T cell dose could delay the onset of toxicity, it did not prevent it.

The most comprehensive study looking at toxicity arising secondary to adoptive transfer of TCR transduced T cells in murine models has been published by Bendle et al. T cells transduced with the OT-1 TCR were adoptively transferred into lethally irradiated C57BL/6 mice followed by the administration of high dose IL-2 10-12 days post transfer. On day 14 post transfer, the recipients of the TCR transduced T cells developed marked cachexia and the majority died as a result of this toxicity. Recipients of mock transduced T cells did not develop any demonstrable toxicity. It was demonstrated in the recipients of OT-1 TCR transduced T cells that there was marked loss of haematopoietic activity within the lymphoid organs and bone marrow. Identical toxicity was demonstrated in OVA transgenic recipients and C57BL/6 recipients (which don't express cognate antigen). Therefore the disease development was not due to an on target toxicity of the TCR transduced T cells. Conversely the adoptive transfer of OT-1 TCR transgenic T cells into C57BL/6 mice did not result in any toxicity and 100% of the recipients survived. Thus the GVHD that was seen following transfer of OT-1

TCR gene modified T cells was not due to cross reactivity of the OT-1 TCR but due to toxicity of the transduced TCR. The authors termed this pathology TCR gene transfer induced GVHD (TI-GVHD). To confirm that TI-GVHD was arising due to mispairing of the introduced OT-1TCR α and β chains, adoptive transfer was carried out using T cells transduced with only the α chain or the β chain alone. TI-GVHD occurred following single chain transduction although with slightly different incidence and kinetics than what was seen following transduction of the complete TCR. Similar experiments were carried out with a further four TCRs (pmel-1, SV40, F5 and Trp-1) and TI-GVHD was seen in all mice using all four of these TCRs. Interestingly the F5-TCR had much lower levels of toxicity than the OT-1 TCR resulting in death in only 20% of recipients. Less toxicity was also demonstrated if a lower dose of IL-2 was administered (31% of mice died) although the survivors developed a chronic GVHD picture. Transduction of a modified OT1-TCR with an additional disulphide bond to reduce mispairing led to a reduced incidence of TI-GVHD but didn't completely prevent it. This paper didn't specifically explore the effects of CD8⁺ transduced T cells versus CD4⁺ T cells. In most cases the transferred population of T cells contained only a low frequency of CD4⁺ T cells. However a small experiment comparing OT-1 TCR transduced CD8⁺ T cells versus OT-1 TCR transduced CD4⁺ T cells demonstrated a higher level of toxicity following transfer of CD4⁺ purified T cells suggesting a dominant role for CD4⁺ T cells in the development of TI-GVHD (196). In studies utilizing human TCR transduction, 7 different TCRs were transduced into 5 virus specific human T cell populations (obtained from healthy donors). These transduced T cells were stimulated with an LCL panel that expressed

the majority of the most common HLA I and II molecules. Neoreactivity was demonstrated in each of the virus specific T cell lines with at least 2 of the 7 introduced TCRs and occurred following introduction of only the α and β chains of the introduced TCRs demonstrating this was occurring as a result of TCR mispairing. Very high frequencies of neoreactive mixed TCR dimers were demonstrated and some were alloreactive whilst others were autoreactive. This was not a specific feature of a specific TCR or of a specific virus specific T cell as it was seen in all of the virus specific T cell lines and with differing introduced alpha and beta chains (290). Although mixed TCR dimer dependent toxicity has been demonstrated in mice models, it has not yet been observed in clinical trials of gene therapy (7) (8).

From the above publications it would suggest that following the introduction of a novel TCR, the incidence of TCR mispairing and potential toxicity arising from this is high. The toxicity seen following adoptive transfer of F5-TCR + CD3 CD4⁺ T cells could be secondary to mispairing of the F5 TCR α and β chains with the endogenous TCR α and β chains generating TCRs of unknown specificities. This hypothesis was supported by the experiments in which CD4⁺ T cells were transduced only with the F5-TCR β chain plus or minus additional CD3 molecules. Following adoptive transfer, there was greatly reduced survival in recipient mice that received F5-TCR β only or F5-TCR β + CD3 CD4⁺ T cells compared to mice receiving mock transduced CD4⁺ T cells. The onset of weight loss with high clinical severity scores occurred at earlier time points in mice receiving F5-TCR β + CD3 CD4⁺ T cells. That CD4⁺ T cells only expressing additional F5-TCR β chains also mediated

toxicity suggesting that this was due to mispairing of the F5-TCR β chain with the endogenous TCR α chain leading to unknown specificities. These novel TCRs had not been subject to stringent negative selection processes in the thymus and it is possible that TCR with high affinity to auto antigens may be generated. The expression level of the mispaired TCR is increased in the presence of additional CD3, greatly potentiating the harmful off target effects. The F5-TCR that has been used in these experiments has been engineered with an additional disulphide bond, which suggests that whilst this strategy can reduce mispairing it does not prevent it completely.

In addition to TCR mispairing there may be additional mechanisms in play mediating the toxic effects of the F5-TCR + CD3 CD4⁺ T cells. Toxicity was not dependent on the presence of cognate NP peptide thus suggesting that toxicity was arising due to an off target effect mediated by the adoptively transferred T cells. Off target effects could potentially arise due to cross reactivity of the highly expressed F5-TCR in the presence of additional CD3. A high level of peptide cross reactivity is an inherent characteristic of antigen recognition by T cells. Mathematical modeling has demonstrated that the T cell pool is not of sufficient size to provide immunity against all possible foreign peptides if TCR are only specific for one single peptide epitope-MHC combination. In fact it has been estimated that each T cell must be able to react to as many as 10^6 structurally similar peptides in order to provide enough immunity against all potential foreign peptides (291) (292). Thus, most, if not all TCR will have the ability to recognize a number of different peptides, which may not always necessarily share strong sequence

homology. In addition, T cells can be activated using peptides that have unrelated sequences to the original peptide on which they were selected on in the thymus (293).

Engineering TCRs to produce high affinity TCR targeting tumour antigens carries with it the risk of inducing cross reactive TCRs. It has been demonstrated that T cells that express very high affinity TCR ($K^d < 1\text{nm}$) start to lose antigen specificity and may become cross or alloreactive (121) (253). In clinical trials there is evidence that transfer of TCR transduced T cells can lead to targeting of normal self antigens eg melan-A specific T cells have been shown to target melanocytes in skin, eye and ear and neurons in MAGE-A3 specific T cells (198). It is possible that the toxicity that is demonstrated in these experiments may be as a direct result of increased functional avidity of the F5-TCR + CD3 CD4+ T cells leading to cross reactivity in a similar manner as that has been described in high affinity TCRs.

Experiments measuring the affinity of TCR-pMHC interaction and generation of high affinity TCR has demonstrated that it is likely that there is a minimum affinity threshold that is required to result in successful stimulation by cognate-pMHC. However above a certain threshold it is likely that no further improvement in sensitivity for pMHC is derived. The range of this maximum and minimum affinity thresholds will also be controlled by whether there is a functional co-receptor interaction. Despite that fact that the class I MHC-CD8 interaction is a low affinity interaction (114) (101), the participation of CD8 to the TCR-pMHC binding can increase the sensitivity of binding of T cell to

cognate pMHC by up to one million fold. This results in CD8⁺ T cells that are able to respond to as few as 1-3 agonist ligands if there is a functional CD8 co-receptor interaction. It is thought that by increasing the affinity of the tumour specific TCR that tolerance to tumour antigens can be broken. However there is the risk that when high affinity TCRs are introduced into CD8⁺ T cells, that T cells are generated that are of such high affinity that they start to lose specificity for their target antigen and start to bind to self antigens non discriminately. As a result, GVHD may arise as a consequence of adoptive transfer of such high affinity TCR.

When generating class I restricted CD4⁺ T cells there is an advantage to introducing a TCR with higher affinity ($K^d < 10\mu\text{M}$) for peptide-class I MHC when there is no additional increase in affinity provided by interaction of a co-receptor. However even when expressed in CD4⁺ T cells there does appear to be an affinity threshold above which T cells become crossreactive with activation occurring in the absence of cognate peptide. This was demonstrated using a HLA-A2 restricted TCR directed against NYESO-1 that had a picomolar affinity (286). Thus even in CD4⁺ T cells there is a risk that engineering high affinity TCRs to improve anti-tumour activity can lead to crossreactivity against structurally related self peptides. For CD4⁺ T cells transduced with a high affinity TCR for cognate pMHC, it would need to cross react with a structurally related self pMHC with an affinity of at least $10\mu\text{M}$ in order to induce autoreactivity whilst for CD8⁺ T cells, the TCR would have to bind to self pMHC with an affinity of only $300\mu\text{M}$ in order to stimulate autoreactivity given the additional interaction of the CD8 co-receptor.

Engels et al generated a TCR with nanomolar affinity, m33, which was generated from the wild type 2C TCR by yeast phage display technology. The 2C TCR recognizes the allogeneic MHC, L^d but also recognizes its cognate peptide, SIY presented by K^b and self peptides, dEV-8 and p2Ca presented by K^b. The affinity of m33-TCR for SIY- K^b was increased by 1000 fold compared to the wild type 2C TCR. In contrast, the affinity for m33-TCR for the self peptide, dEV8-K^b demonstrated only a two fold increase in affinity compared to wild type. Murine CD8⁺ and CD4⁺ T cells were transduced with the m33 TCR. CD8⁺ T cells transduced with m33 were able to lyse cancer cells overexpressing dEV-8 whilst 2C TCR only lysed SIY overexpressing cells. CD4⁺ T cells transduced with the m33 TCR did not respond to dEV-8-K^b but were able to make efficient responses to SIY- K^b leading to IFN- γ production and target cell lysis. CD4⁺ T cells transduced with the wild type 2C TCR showed only low reactivity for SIY. Following adoptive transfer in vivo these m33 CD8⁺ T cells disappeared very rapidly post transfer into a syngeneic H2^b hosts. These CD8⁺ T cells expressed a TCR that had high affinity for a ubiquitously expressed self antigen and as a result were deleted by peripheral tolerance mechanisms. There was no evidence of generation of GVHD following transfer of CD8⁺ T cells. CD8⁺ T cells transduced with the lower affinity 2C TCR were able to survive long term in vivo. Contrastingly, CD4⁺ T cells transduced with m33 showed anti-tumour effects in vivo in the absence of co-transfer of antigen specific CD8⁺ T cells and were able to persist in vivo for greater than 3 months. CD4⁺ T cells transduced with m33

TCR and additional CD8 molecules underwent rapid deletion *in vivo* following adoptive transfer (294).

It is not clear why in this model system, the expression of a high affinity TCR in CD8⁺ T cells led to deletion and not GVHD. It might be related to the wide spread expression of the self antigen to which the TCR was specific. The strength of the pMHC binding may have overwhelmed the introduced CD8⁺ T cells leading to fratricide or to antigen induced cell death or may be due to the high affinity TCR binding to self antigen with very strong and persistent binding. When a cross reactive T cell is specific for an antigen that has more tissue restricted expression the T cells may be able to persist and it may lead to localized tissue destruction. TCR affinity or avidity thus plays an important role in recognition of structurally similar peptides to that of the cognate antigen and thus is one of the major determinants of TCR cross reactivity. It is clear that an increase in affinity of a TCR can lead to an increase in self reactivity or loss of specificity. Similarly it would be expected that the increase in surface expression of TCR by the provision of additional CD3 leading to enhanced functional avidity could potentially lead to increased cross reactivity for self peptide and thus targeting of normal tissues and GVHD following adoptive transfer. Ordinarily self reactive T cells will be controlled by peripheral tolerance mechanisms such as ignorance due to low level expression of self antigen when presented in a non inflammatory environment. T cells that bind to self-antigen in the periphery may also become anergic if self antigen is presented by professional APCs that lack co-stimulatory molecules. In addition, potentially auto reactive T cells will be negatively regulated by

suppressive CD4⁺ T regulatory cells. It may be that when a TCR is expressed at a supra physiological level, then the normal tolerance controls are broken. The effects of cross reactivity may be even further enhanced in a model where a large number of monoclonal T cells are transferred into a lymphopaenic environment, which may lead to much higher rates of targeting of self antigens than would ordinarily be seen. At this level, the normal checkpoints of peripheral tolerance may be overwhelmed particularly if the cross reactive T cell is expressed at a very high level. In addition, following irradiation, the numbers of recipient T regulatory CD4⁺ T cells that would normally be controlling self reactive T cells are markedly depleted.

From clinical trials there is increasing recognition of the potential side effects related to recognition of self antigens by the TCR transduced T cells. The DMF4 TCR was the original TCR utilized in the first clinical trial of TCR transduced T cells in melanoma patients. It is specific for a differentiation antigen, MART-1 which is upregulated on the surface of melanoma cells. DMF-4 TCR is specific for an epitope of MART-1 presented by HLA-A2 and has relatively low affinity for it's cognate pMHC (K^d 170 μ M). No evidence of autoimmunity was described in the clinical trial using the DMF4 TCR (7). A second generation TCR was derived from the original DMF4 TCR, called DMF5, which was engineered to be of higher avidity and it had demonstrable in vitro activity when transduced into CD4⁺ T cells as well as CD8⁺ T cells (8). The affinity of the DMF5 TCR (K^d 40 μ M) was higher than DMF4. The overall objective response rates in clinical trials using the DMF5 TCR were higher than those using DMF4 TCR (30% versus 13%) but patients treated

with DMF5 TCR experienced a marked spike of IFN- γ production following transfer of TCR transduced T cells and they developed serious skin rashes 3-5 days post transfer. A high rate of anterior uveitis, hearing loss and dizziness were also reported, thought to be secondary to targeting of self antigen in the periphery by the high affinity TCR.

Finally, it was hypothesized that addition of an excess of CD3 molecules may also lead to upregulation of the endogenous TCR. The high expression level of the endogenous TCR may break peripheral tolerance to self antigens leading to auto reactivity and GVHD. This hypothesis was supported by the finding that the adoptive transfer of CD3 only CD4⁺ T cells (transduced only with additional CD3 but no additional TCR) into irradiated recipients led to an excess of mortality above what was seen following adoptive transfer of mock transduced CD4⁺ T cells. 50% of mice receiving CD3 only CD4⁺ T cells developed rapid cachexia and high clinical severity scores. Whilst higher levels of toxicity were seen following adoptive transfer of F5-TCR + CD3 CD4⁺ T cells, this would support the hypothesis that some adverse effects mediated by the adoptively transferred populations arose secondary to upregulation of the endogenous TCR. The higher expression level of the endogenous TCR and thus the enhanced functional avidity of these cells may lead to breaking of normal peripheral tolerance mechanisms and generation of autoreactive T cells targeting normal self tissues. The peripheral T cell repertoire is determined during thymic development during which T cells, which recognize self-peptide and MHC with low affinity undergo positive selection whilst those that recognize self peptide and MHC with high affinity

are deleted during negative selection. This leaves a T cell repertoire that has low affinity for self-antigen but high affinity for foreign antigen. In normal steady state conditions, peripheral tolerance mechanisms prevent self-reactivity. It is feasible that when endogenous TCR expression is markedly increased to supra physiological levels in the presence of additional CD3 that the self-reactive endogenous TCR will bind to self antigen with higher affinity.

The amount of inflammation and the amount of lymphodepletion of host haematopoiesis appear to be important drivers of toxic effects mediated by adoptively transferred T cell populations. Reduced irradiation doses result in markedly reduced inflammation and tissue damage and also reduced expansion of the adoptively transferred T cells. Tissue damage produced at high irradiation doses leads to release of danger signals, which leads to activation of professional APCs, which express higher levels of MHC and co-stimulatory molecules. In addition, the presence of high avidity TCR and the presence of strong co-stimulation may be able to overrule CD4⁺ T regulatory suppression (295). This would lead to enhancement of any potential targeting of self-tissues by the adoptively transferred cells. Auto reactivity may be promoted as a result of epitope spreading which may arise in the context of acute inflammation induced by irradiation. The immune response of the transduced T cells may originally target the NP peptide but in the context of inflammation, the specificity of the immune response may spread to involve self-epitopes. This may then prime and activate other autoreactive T cells, recruiting them into the immune response. It is also possible during acute inflammation that cryptic or sequestered epitopes are released from damaged

tissues, thus further activating the immune response (296). In addition molecular mimicry may lead to activation of cross reactive T cells that respond to both the NP peptide and self epitopes.

Toxicity was completely prevented if F5-TCR + CD3 CD4⁺ T cells were adoptively transferred into mice that had been irradiated with only 4 Gy, leading to 100% survival in this group. However the toxicity that was seen at the higher doses of irradiation was not purely due to direct effects of radiation doses as demonstrated by 100% survival seen in mice irradiated with 5.5 Gy who received adoptive transfer of mock transduced CD4⁺ T cells. At a lower dose of irradiation, the tissue damage mediated by the irradiation is less and in addition there is significantly lower levels of expansion of the adoptively transferred T cells. Similarly in the publication by Bendle et al, mice that received lower doses of IL-2 post adoptive transfer had much lower levels of toxicity and superior overall survival than mice given high dose IL-2 (196). The higher dose of IL-2 used in these experiments would promote much higher levels of expansion of the adoptively transferred cells and thus potentiate any adverse effects mediated by the transferred T cell populations.

No publications have yet compared whether adoptive transfer of class I restricted CD4⁺ transduced T cells lead to higher levels of toxicity than CD8⁺ TCR transduced T cells. Whilst adverse effects were seen in mice receiving CD8⁺ T cells transduced with F5-TCR and CD3 they were not as severe as that observed following adoptive transfer of CD4⁺ T cells transduced with F5-TCR and CD3. Median survival in mice receiving F5-TCR+CD3 CD4⁺ T cells

was 13.5 days compared to 15.5 days in mice receiving F5-TCR+ CD3 CD8+ T cells (p=ns). Whilst there was 100% mortality seen in recipients of F5-TCR+CD3 CD4+ T cells, 20% of the mice receiving F5-TCR+CD3 CD8+ T cells survived long term with no adverse events seen. It is possible that CD4+ T cells have inherently higher autoreactive potential than CD8+ T cells and this may be a function of class I restricted CD4+ T cells, as class I restricted CD4+ T cells may be able to respond to antigen presented by non professional APCs. Ordinarily CD4+ T cells recognize antigen presented by class II MHC on professional APCs. Whilst class I restricted TCR can bind antigen presented by non-professional APCs on class I MHC, the majority of CD8+ T cells require priming via cross presentation of antigen by class I MHC on professional APCs following licensing of the APCs via CD4+ T cell help. Thus whilst CD8+ T cells require the presence of a CD4+ T cell to prime the APC to make antigen specific responses, particularly in the context of a non-inflammatory antigen, class I restricted CD4+ T cell may not have such stringent activation requirements. In addition, in the context of an introduced TCR that is very highly expressed there may be less reliance on the expression of co-stimulatory molecules by the APC for activation of the CD4+ T cell. There is no evidence that the CD4 or CD8 $\alpha\beta$ co-receptor plays an inhibitory role in T cell activation and thus it seems unlikely that it is the lack of CD4 co-receptor interaction that is leading to increased auto reactivity of the CD4+ T cells. It may be that CD4+ T cells have a higher autoreactive potential than CD8+ T cells or that the expression level of the TCR is higher within the CD4+ T cell population.

Whilst improvements to TCR functional avidity can improve anti tumour effects, these experiments have highlighted that this may carry with it an increased risk of inducing targeting of normal self tissues by the adoptively transferred cells. How to separate increased tumour protection from potential harmful side effects of the transduced T cells will prove challenging. Further work is needed to delineate the exact nature of the toxicity mediated by CD4+ T cells co-transduced with additional CD3 and also the role of CD4+ versus CD8+ T cells in mediating toxic effects post adoptive transfer. In addition, this was a model system that used a target antigen that is not a self-peptide. It is possible that even higher toxic effects may be noted when adoptively transferring T cells that are specific for a tumour peptide that is also expressed in normal self tissues. It highlights the risk that may arise following adoptive transfer of TCR transduced T cells, particularly when utilizing high avidity TCRs or forcing supra physiological levels of TCR expression.

CHAPTER 6 – CONCLUSIONS AND FUTURE WORK

These experiments have explored whether the functional avidity of a CD4⁺ T cell transduced with a class I restricted TCR can be improved by the transduction of additional CD3. Given that one of the main determinants of TCR avidity is the density of TCR expression, increasing the transduced TCR expression is a very simple way to enhance the avidity of the TCR. Co-transduction of CD3 plus TCR provides an excess of CD3 molecules so that competition between the introduced and the endogenous TCR for binding to CD3 is removed leading to higher levels of surface expression of the introduced TCR. In vitro, CD4⁺ T cells co-transduced with F5-TCR and CD3 did have enhanced functional avidity, demonstrating a two fold increase in sensitivity to peptide when assessing cytokine production and proliferation responses following stimulation with specific peptide. The co-transduction of F5-TCR and CD3 did not improve the functional avidity of a CD4⁺ T cell to that of a CD8⁺ T cell expressing F5-TCR only and it may be that in order to achieve this, additional CD8 co-receptor would need to be co-transduced with the F5-TCR.

In vivo, F5-TCR and CD3 CD4⁺ T cells reduced the volume of tumour burden when adoptively transferred into irradiated syngeneic mice challenged with EL4-NP tumours. The CD4⁺ T cells were transferred in isolation, without tumour specific CD8⁺ T cells and thus tumour reduction that was demonstrated was thought to be due entirely to the effects of the CD4⁺ T cells. The exact mechanism of tumour protection was not explored in this

model. CD4⁺ T cells transduced with F5-TCR alone were not able to provide any degree of tumour protection and this was thought to be a result of the impaired functional avidity of the F5-CD4⁺ T cell when TCR expression was at a lower level in the absence of additional CD3.

Following adoptive transfer *in vivo*, it was demonstrated that the expansion and persistence of the F5-TCR + CD3 CD4⁺ T cells was superior than that of F5-TCR CD4⁺ T cells alone. In addition, F5-TCR + CD3 CD4⁺ T cells were able to traffic to the tumour bed at faster rates and were present at the tumour bed in higher numbers. This superior *in vivo* expansion and trafficking led to more effective tumour protection post adoptive transfer.

Unexpectedly, mice receiving F5-TCR + CD3 CD4⁺ T cells developed marked toxicity that was not seen in mice receiving F5-TCR CD4⁺ T cells or mock transduced CD4⁺ T cells. This toxicity was thought to have arisen as a direct result of the F5-TCR + CD3 CD4⁺ T cells. This toxicity was not dependent on the presence of cognate peptide and thus could be due to an off target effect of the introduced TCR. It was hypothesized that the toxicity was arising due to the very high surface expression of the introduced TCR leading to generation of autoreactive T cells that could circumvent normal tolerance mechanisms. In addition, toxicity was seen when additional CD3 was introduced into the CD4⁺ T cell even in the absence of an introduced TCR. It was hypothesized that the provision of an excess of CD3 led to upregulation of the endogenous TCR. The endogenous TCR when expressed at a supraphysiological level, may be breaking normal tolerance mechanisms and targeting self antigen

leading to GVHD. There may also be a role for TCR mispairing in this model as demonstrated by the very fast onset of toxicity that was seen in mice that received adoptive transfer of CD4⁺ T cells transduced with the F5-TCR β chain alone.

This project did not explore fully the mechanism by which CD4⁺ T cells eradicated tumour in vivo and also the mechanism by which toxicity arose in this model. Further experiments should be carried out to explore the exact role of CD4⁺ T cell help in vivo. Reduction in tumour burden was seen when F5-TCR + CD3 CD4⁺ T cells were transferred in isolation. It is not clear if these effects were due to direct cytotoxicity of the CD4⁺ T cells or whether the CD4⁺ T cells were recruiting innate recipient immune cells such as NK cells or macrophages to eradicate the tumour. In sub lethally irradiated mice where recipient haematopoiesis has been greatly reduced, it would suggest that direct cytotoxicity by adoptively transferred CD4⁺ T cells is the main mechanism of tumour eradication in these models. To explore this in more detail, adoptive challenge and tumour challenge could be carried out in RAG -/- recipient mice which would determine if there was any role for endogenous CD4⁺, CD8⁺ or B cells. Alternatively anti-CD8 or anti NK1.1 antibodies could be administered to deplete endogenous CD8 and NK T cells. It would also be preferable to carry out tumour challenge and adoptive transfer in non irradiated controls to ensure that there was no effect of the irradiation itself on toxicity or upon tumour protection. In vivo cytotoxicity assays to demonstrate whether CD4⁺ T cells are cytotoxic have been technically difficult given the high and rapid onset of toxicity seen following adoptive transfer of the F5-TCR

+ CD3 CD4⁺ T cells. Alternatively, In vitro cytotoxicity assays to explore whether F5-TCR + CD3 CD4⁺ T cells are capable of direct toxicity to peptide loaded splenocytes or tumour cells in vitro could be performed. Tumour challenge experiments and adoptive transfer could also be carried out using CD4⁺ T cells derived from perforin ^{-/-} or granzyme ^{-/-} mice. A reduction in tumour killing in the absence of these cytotoxic mechanisms would suggest that F5-TCR + CD3 CD4⁺ T cells do mediate tumour protection at least in part by cytotoxic mechanisms. To determine if there is any role for IFN- γ or TNF- α at mediating tumour rejection, neutralizing antibodies directed against these cytokines could be administered following adoptive transfer.

A comparison of the adoptive transfer of F5-TCR + CD3 CD4⁺ T cells and F5-TCR + CD3 CD8⁺ T cells demonstrated lower levels of toxicity and superior overall survival in mice receiving CD8⁺ transduced T cells compared to CD4⁺ T cells. This requires further investigation to try to determine the mechanism of toxicity and why CD4⁺ T cells co-transduced with TCR and CD3 would cause more harmful effects than CD8⁺ T cells transduced with TCR and CD3. It may be that CD8⁺ T cell responses are more tightly regulated than CD4⁺ T cells or that CD4⁺ T cells, by virtue of their helper functions may lead to activation of a broad range of effector cells that are causing non specific tissue damage.

Ex vivo experiments to try to reexpand the adoptively transferred T cells or to demonstrate cytokine production to demonstrate their function following in vivo transfer and to determine whether the F5-TCR CD4⁺ T cells and F5-TCR

+ CD3 CD4⁺ T cells demonstrate any autoreactivity in vitro may add more information regarding the differing functions of the F5-TCR only or F5-TCR + CD3 CD4⁺ T cells. Cytokine production ex vivo could be measured by luminex assays following stimulation with NP-peptide, to determine if the adoptively transferred populations maintain the same cytokine production profile that had been demonstrated in vitro prior to adoptive transfer. Potentially, the adoptively transferred T cells may have altered function following transfer in vivo. In vitro functional assays using a syngeneic MLR to stimulate the ex vivo T cells using syngeneic APCs may demonstrate reactivity against syngeneic cells that do not express the cognate peptide and may demonstrate autoreactivity of the adoptively transferred cells.

A comparison of CD4⁺ T cells transduced with F5-TCR and CD3 with CD4⁺ T cells transduced with F5-TCR and CD8 would provide information about the the degree to which both techniques improves functional avidity. From these experiments it suggests that the provision of additional CD3 did not improve the functional avidity of the F5-TCR CD4⁺ T cell to that of a F5-TCR CD8⁺ T cell. Potentially, the transduction of CD8 plus the F5-TCR could generate CD4⁺ T cells that have the same functional avidity of F5-TCR CD8⁺ T cell so an experiment comparing the in vitro function of F5-TCR + CD3 CD4⁺, F5-TCR + CD8 CD4⁺ T cells and F5-TCR CD8⁺ T cell populations could be carried out. Adoptive transfer of F5-TCR CD8 CD4⁺ T cells could be carried out to determine if the F5-TCR CD8 CD4⁺ T cells mediate the same level of toxicity as that of a F5-TCR CD3 CD4⁺ T cell.

Tissue sections could be assessed to demonstrate definitively whether there was evidence of graft versus host disease in mice that died of toxicity following adoptive transfer. The GVHD that has been described post adoptive transfer has been shown to have a predilection for targeting of haematopoietic tissues such as spleen bone marrow resulting in marked reduction of normal haematopoiesis. Serum samples could be obtained from the recipients of adoptively transferred T cells and analyzed for production of differing cytokines to try to determine if there were any differences in cytokines produced in vivo and potentially whether they had a causative effect in the toxicity mediated by the transduced CD4⁺ T cells. Bendle et al demonstrated that GVHD secondary to adoptively transferred TCR transduced T cells was prevented if T cells from IFN- γ knockout mice were transduced and used for adoptive transfer. This suggested that the production of IFN- γ by the adoptively transferred T cells was an important mediator toxic effects of the transduced T cells (196). T cells derived from IFN- γ ^{-/-} knockout mice or from granzyme or perforin ^{-/-} knockout mice could be used for transduction with the F5-TCR and CD3 to determine if reduced toxicity is seen when these effector mechanisms are absent from the transduced T cells.

F5-TCR + CD3 CD4⁺ T cells were predominantly of a Th1 phenotype and thus it would be interesting to explore whether toxicity was seen with the same cells if they were skewed to a Th2 phenotype prior to adoptive transfer. Transduced T cells could be cultured in the presence of IL-4 to polarize these cells to a Th2 phenotype prior to adoptive transfer to determine whether this has any effect on the level of toxicity that is seen and also on the degree of

tumour protection that is seen. Th1 and Th17 CD4⁺ T cells appear to be the predominant cell type at mediating autoimmunity in vivo whilst Th2 CD4⁺ T cells do not appear to induce autoimmunity in most model systems. Given the plasticity of CD4⁺ T cell subsets, it is possible that the Th2 polarized CD4⁺ T cells may not maintain a stable phenotype and that following transfer into an inflammatory environment may switch back to a Th1 phenotype.

To confirm the results of this series of experiments, tumour challenge should be carried out with CD4⁺ T cells transduced with a differing range of TCRs to ensure that these results are not as a result of a peculiarity of the F5-TCR only. The WT1-TCR is a fully CD8 dependent class I restricted TCR which is not functional when expressed in CD4⁺ T cells in isolation. WT1 is a transcription factor involved in growth and differentiation and is expressed within the kidneys, gonads, uterus and mesothelium. WT1 is overexpressed in Acute Myeloid Leukaemia, myelodysplasia, Acute Lymphoblastic Leukaemia and Chronic Myeloid Leukaemia and also in a number of solid organ malignancies. To fully explore this, an in vivo system using a murine leukaemia model where the leukaemia cells expressed WT1 could be utilized. Adoptive transfer of WT1-TCR + CD3 CD4⁺ T cells targeting WT1 would allow exploration of toxicity using a different TCR and also would represent a more physiological system where the tumour antigen is also expressed by normal tissues.

Given that increased toxicity is seen when CD3 is transduced into polyclonal CD4⁺ T cells in isolation it was hypothesized that the endogenous TCR was

being upregulated. In order to properly assess the level of expression of the endogenous TCR following transduction of CD3 only, cells could be stained for a number of common V β or V α regions to look for upregulation of TCRs that express that particular variable region. This could be compared to untransduced T cells to see if there is any upregulation of the endogenous TCR. Alternatively, T cells could be sorted to produce a pure population of T cells that express a particular V β subtype prior to transduction of CD3 and this may show quite clearly if there is an increase in surface expression following transduction of additional CD3 molecules.

In addition to transducing CD4⁺ T cells with F5-TCR β chain, a vector could be generated that expressed only the F5-TCR α chain. CD4⁺ T cells could be transduced with the F5-TCR α chain plus or minus additional CD3. Given the effect of transducing F5-TCR β chain plus or minus additional CD3, it would be expected that the F5-TCR α would mispair with the endogenous TCR β chains leading to neoreactive T cells.

To further explore the effects of TCR mispairing, a monoclonal population of CD4⁺ T cells, e.g OT-II T cells, could be transduced with the F5-TCR. In this situation, only 2 novel TCR specificities can be generated and thus the degree of autoimmunity seen in this situation is solely dependent on the mispairing of the F5-TCR. Thus the potential for generating autoreactive T cells should be much lower than that which would be seen when transducing a polyclonal CD4⁺ T cell population.

These experiments have demonstrated severe toxicity that has arisen as a direct consequence of the adoptively transferred CD4⁺ T cells. TCR engineering to enhance anti-tumour potential of transduced T cells has to be carefully balanced with the potential risk of generating lethal autoimmunity. There needs to be clearer understanding of the mechanism by which the CD4⁺ T cells mediate toxicity and to what extent TCR mispairing and upregulation of the endogenous TCR played a role in the toxic effects of the adoptively transferred CD4⁺ T cells. Whether CD4⁺ T cells are shown to be inherently more autoreactive than CD8⁺ T cells will also have important implications if CD4⁺ T cells expressing high affinity TCRs are to be utilized in clinical trials. The strategy of increasing TCR surface expression by co-transduction of CD3 may be limited if a byproduct of this technique is to increase the surface expression of the endogenous TCR and thus generate autoreactive T cells. In addition, if TCR mispairing plays a major role in toxicity then this would suggest that techniques to prevent TCR mispairing such as introduction of an additional disulphide bond can't completely prevent TCR mispairing. Strategies that downregulate the expression of the endogenous TCR such as zinc finger nucleases may be a safer technique to utilize. This also highlights the need to have a suicide gene mechanism in place in transduced T cells that will be used for adoptive transfer so that any autoreactive T cells can be efficiently and quickly deleted should GVHD arise in vivo.

APPENDIX 1 – LIST OF ABBREVIATIONS

TCR – T cell Receptor

PBMC – Peripheral Blood Mononuclear Cell

APC – Antigen Presenting Cell

LTR – Long Terminal Repeat

DC – Dendritic Cell

HSC – Haematopoietic Stem Cell

Ii – Invariant Chain

CDR – Complementarity Determining Region

HSV-TK – Herpes Simplex Virus Thymidine Kinase

DLI – Donor Lymphocyte Infusion

GVHD – Graft Versus Host Disease

CID – Chemical Inducer of Dimerization

IL-2R- γ – Interleukin 2 receptor gamma chain

Tregs – CD4⁺ T regulatory cells

Tfh - T follicular helper CD4⁺ T cell

CTL – Cytotoxic T Lymphocyte

PTLD – Post transplant lymphoproliferative disorder

Ph-Eco – Phoenix Ecotrophic cell

BMDC – Bone Marrow derived Dendritic Cell

ER – Endoplasmic Reticulum

TAP – Transporter associated with antigen processing

MIIC – MHC class II compartment

CLIP – Class II associated peptide

HEV – High endothelial venule

CAR – Chimeric Antigen receptors

APPENDIX 2

Sequence of pMP71-F5 α -2A-F5 β

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1 AGCATCGTTC TGTGTTGTCT CTGTCTGACT GTGTTTCTGT ATTTGTCTGA
51 AAATTAGCTC GACAAAGTTA AGTAATAGTC CCTCTCTCCA AGCTCACTTA
101 CAGGCGGCCG CGCCACCATG AACTATTCTC CAGCTTTAGT GACTGTGATG
151 CTGTTTGTGT TTGGGAGGAC CCATGGAGAC TCAGTAACCC AGATGCAAGG
201 TCAAGTGACC CTCTCAGAAG ACGACTTCCT ATTTATAAAC TGTACTTATT
251 CAACCACATG GTACCCGACT CTTTTCTGGT ATGTCCAATA TCCTGGAGAA
301 GGTCCACAGC TCCTTTTGAA AGTCACAACA GCCAACAACA AGGGAATCAG
351 CAGAGGTTTT GAAGCTACAT ATGATAAAGG AACACGTCC TTCCACTTGC
401 AGAAAGCCTC AGTGCAGGAG TCAGACTCTG CTGTGTACTA CTGTGTTCTG
451 GGTGATCGAC AGGGAGGCAG AGCTCTGATA TTTGGAACAG GAACCACGGT
501 ATCAGTCAGC CCCAACATCC AGAACCCAGA ACCCGCGGTG TACCAGCTGA
551 AGGACCCAG AAGCCAGGAC AGCACCTGT GCCTGTTTAC CGACTTCGAC
601 AGCCAGATCA ACGTGCCCAA GACAATGGAA AGCGGCACCT TCATCACCAG
651 CAAGTGCGTG CTGGACATGA AGGCTATGGA CAGCAAGAGC AACGGCGCCA
701 TCGCCTGGTC CAACCAGACC TCCTTCACAT GCCAAGACAT CTTCAAAGAG
751 ACCAACGCCA CCTACCCAG CAGCGACGTG CCCTGCGATG CCACTCTCAC
801 CGAGAAGAGC TTCGAGACCG ACATGAACCT GAACTTCCAG AACCTGAGCG
851 TGATGGGCCT GAGAATCCTG CTCCTGAAAG TGGCCGGCTT CAACCTGCTG
901 ATGACCCTGC GGCTCTGGAG TTCTGGCAGC GGCCTACCA ACTTCAGCCT
951 GCTGAAGCAG GCCGGCGACG TGGAGGAAAA CCCTGGGCCC ATGGCCCCC
1001 GGCTCCTTTT CTGTCTGGTT CTTTGCTTCT TGAGAGCAGA ACCAACAAAT
1051 GCTGGTGTCA TCCAAACACC TAGGCACAAG GTGACAGGGA AGGGACAAGA
1101 AGCAACTCTG TGGTGTGAGC CAATTTAGG ACATAGTGCT GTTTTCTGGT
1151 ACAGACAGAC CATTGTGCAG GGCCTGGAGT TCCTGACTTA CTTTCGAAAT
1201 CAAGCTCCTA TAGATGATTC AGGGATGCCC AAGGAACGAT TCTCAGCTCA
1251 GATGCCCAAT CAGTCGCACT CAACTCTGAA GATCCAGAGC ACGCAACCCC
1301 AGGACTCAGC GGTGTATCTT TGTGCAAGCA GCTCCCGGAC TGGGGGGCAT
1351 GCTGAGCAGT TCTTCGGACC AGGGACACGA CTCACCGTCC TCGAGGACCT
1401 GCGGAACGTG ACCCCCCCA AGGTGTCCCT GTTCGAGCCC AGCAAGGCCG
1451 AGATCGCCAA CAAGCAGAAA GCCACACTGG TCTGTCTGGC TAGGGGCTTC
1501 TTCCCCGACC ACGTGGAGCT GTCTTGGTGG GTCAACGGCA AAGAAGTCCA
1551 TAGCGGCGTC TGCACCGACC CTCAGGCTTA CAAAGAGAGC AACTACTCCT
1601 ACTGCCTGAG CAGCCGGCTG AGAGTGAGCG CCACCTTCTG GCACAACCCC
1651 CGGAACCACT TCCGGTGCCA GGTGCAGTTC CACGGCCTGA GCGAAGAGGA
1701 CAAGTGGCCT GAGGGCTCCC CCAAGCCCGT GACCCAGAAC ATCAGCGCCG
1751 AGGCCCTGGG CAGAGCCGAC TGCGGCATCA CCAGCGCCAG CTACCACCAG
1801 GCGGTGCTGT CCGCCACCAT CCTGTACGAG ATCCTGCTGG GCAAGGCCAC
1851 ACTGTACGCC GTGCTGGTGT CCGGCCTGGT CCTGATGGCT ATGGTGAAGA
1901 AGAAGAACAG CTGATGAGTC GACTGTACAA GTAAGAATTC GGATCCAAGC
1951 TTAGGCCTGC TCGCTTTCTT GCTGTCCCAT TTCTATTAAA GGTTCCTTTG
2001 TTCCCTAAGT CCAACTACTA AACTGGGGGA TATTATGAAG GGCCTTGAGC
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2301 CCGGCTCAGG GCCAAGAACA GTTGGAACAG CAGAATATGG GCCAAACAGG
2351 ATATCTGTGG TAAGCAGTTC CTGCCCCGGC TCAGGGCCAA GAACAGATGG
2401 TCCCCAGATG CGGTCCCGCC CTCAGCAGTT TCTAGAGAAC CATCAGATGT
2451 TTCCAGGGTG CCCCAAGGAC CTGAAATGAC CCTGTGCCTT ATTTGAAC
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2501	ACCAATCAGT	TCGCTTCTCG	CTTCTGTTTCG	CGCGCTTCTG	CTCCCCGAGC
2551	TCAATAAAAG	AGCCCACAAC	CCCTCACTCG	GCGCGCCAGT	CCTCCGATAG
2601	ACTGCGTCGC	CCGGGGTACC	CGTATTCCCA	ATAAAGCCTC	TTGCTGTTTG
2651	CATCCGAATC	GTGGACTCGC	TGATCCTTGG	GAGGGTCTCC	TCAGATTGAT
2701	TGACTGCCCA	CCTCGGGGGT	CTTTCATTCT	CGAGAGCTTT	GGCGTAATCA
2751	TGGTCATAGC	TGTTTCCTGT	GTGAAATTGT	TATCCGCTCA	CAATTCCACA
2801	CAACATACGA	GCCGGAAGCA	TAAAGTGTA	AGCCTGGGGT	GCCTAATGAG
2851	TGAGCTAACT	CACATTAATT	GCGTTGCGCT	CACTGCCCCG	TTTCCAGTCG
2901	GGAAACCTGT	CGTGCCAGCT	GCATTAATGA	ATCGGCCAAC	GCGCGGGGAG
2951	AGGCGGTTTTG	CGTATTGGGC	GCTCTTCCGC	TTCCTCGCTC	ACTGACTCGC
3001	TGCGCTCGGT	CGTTCGGCTG	CGGCGAGCGG	TATCAGCTCA	CTCAAAGGCG
3051	GTAATACGGT	TATCCACAGA	ATCAGGGGAT	AACGCAGGAA	AGAACATGTG
3101	AGCAAAAGGC	CAGCAAAAGG	CCAGGAACCG	TAAAAAGGCC	GCGTTGCTGG
3151	CGTTTTTCCA	TAGGCTCCGC	CCCCCTGACG	AGCATCACAA	AAATCGACGC
3201	TCAAGTCAGA	GGTGGCGAAA	CCCGACAGGA	CTATAAAGAT	ACCAGGCGTT
3251	TCCCCCTGGA	AGCTCCCTCG	TGCGCTCTCC	TGTTCCGACC	CTGCCGCTTA
3301	CCGGATACCT	GTCCGCCTTT	CTCCCTTCGG	GAAGCGTGGC	GCTTCTCAA
3351	TGCTCACGCT	GTAGGTATCT	CAGTTCGGTG	TAGGTCGTTT	GCTCCAAGCT
3401	GGGCTGTGTG	CACGAACCCC	CCGTTTCAGCC	CGACCGCTGC	GCCTTATCCG
3451	GTAACATATCG	TCTTGAGTCC	AACCCGGTAA	GACACGACTT	ATCGCCACTG
3501	GCAGCAGCCA	CTGGTAACAG	GATTAGCAGA	GCGAGGTATG	TAGGCGGTGC
3551	TACAGAGTTC	TTGAAGTGGT	GGCCTAACTA	CGGCTACACT	AGAAGGACAG
3601	TATTTGGTAT	CTGCGCTCTG	CTGAAGCCAG	TTACCTTCGG	AAAAAGAGTT
3651	GGTAGCTCTT	GATCCGGCAA	ACAAACCACC	GCTGGTAGCG	GTGGTTTTTT
3701	TGTTTGCAAG	CAGCAGATTA	CGCGCAGAAA	AAAAGGATCT	CAAGAAGATC
3751	CTTTGATCTT	TTCTACGGGG	TCTGACGCTC	AGTGGAACGA	AAACTCACGT
3801	TAAGGGATTT	TGGTCATGAG	ATTATCAAAA	AGGATCTTCA	CCTAGATCCT
3851	TTTAAATTAA	AAATGAAGTT	TTAAATCAAT	CTAAAGTATA	TATGAGTAAA
3901	CTTGGTCTGA	CAGTTACCAA	TGCTTAATCA	GTGAGGCACC	TATCTCAGCG
3951	ATCTGTCTAT	TTCGTTTCATC	CATAGTTGCC	TGACTCCCCG	TCGTGTAGAT
4001	AACTACGATA	CGGGAGGGCT	TACCATCTGG	CCCCAGTGCT	GCAATGATAC
4051	CGCGAGACCC	ACGCTCACCG	GCTCCAGATT	TATCAGCAAT	AAACCAGCCA
4101	GCCGGAAGGG	CCGAGCGCAG	AAGTGGTCCT	GCAACTTTAT	CCGCCTCCAT
4151	CCAGTCTATT	AATTGTTGCC	GGGAAGCTAG	AGTAAGTAGT	TCGCCAGTTA
4201	ATAGTTTGCG	CAACGTTGTT	GCCATTGCTG	CTGGCATCGT	GGTGTACGCG
4251	TCGTCGTTTG	GTATGGCTTC	ATTAGCTCC	GGTTCCCAAC	GATCAAGGCG
4301	AGTTACATGA	TCCCCCATGT	TGTGCAAAAA	AGCGGTTAGC	TCCTTCGGTC
4351	CTCCGATCGT	TGTCAGAAGT	AAGTTGGCCG	CAGTGTTATC	ACTCATGGTT
4401	ATGGCAGCAC	TGCATAATTC	TCTTACTGTC	ATGCCATCCG	TAAGATGCTT
4451	TTCTGTGACT	GGTGAGTACT	CAACCAAGTC	ATTCTGAGAA	TAGTGTATGC
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4601	AAAACCTCTCA	AGGATCTTAC	CGCTGTTGAG	ATCCAGTTCG	ATGTAACCCA
4651	CTCGTGCACC	CAACTGATCT	TCAGCATCTT	TTACTTTCAC	CAGCGTTTCT
4701	GGGTGAGCAA	AAACAGGAAG	GCAAAATGCC	GCAAAAAAGG	GAATAAGGGC
4751	GACACGGA	TGTTGAATAC	TCATACTCTT	CCTTTTTTCAA	TATTATTGAA
4801	GCATTTATCA	GGGTATTATT	CTCATGAGCG	GATACATATT	TGAATGTATT
4851	TAGAAAAATA	AACAAATAGG	GGTTCGCGC	ACATTTCCCC	GAAAAAGTGCC
4901	ACCTGACGTC	TAAGAAACCA	TTATTATCAT	GACATTAACC	TATAAAAAATA
4951	GGCGTATCAC	GAGGCCCTTT	CGTCTTCAAG	CTGCCTCGCG	CGTTTCGGTG
5001	ATGACGGTGA	AAACCTCTGA	CACATGCAGC	TCCCGGAGAC	GGTCACAGCT
5051	TGTCTGTAAG	CGGATGCCGG	GAGCAGACAA	GCCCGTCAGG	GCGCGTCAGC
5101	GGGTGTTGGC	GGGTGTCGGG	GCGCAGCCAT	GACCCAGTCA	CGTAGCGATA
5151	GTTACTATGC	GGCATCAGAG	CAGATTGTAC	TGAGAGTGCA	CCATATGCGG
5201	TGTGAAATAC	CGCACAGATG	CGTAAGGAGA	AAATACCGCA	TCAGGCGCCA
5251	TTGCGCATTC	AGGCTGCGCA	ACTGTTGGGA	AGGGCGATCG	GTGCGGGCCT

5301 CTTGCTATT ACGCCAGCTG GCGAAAGGGG GATGTGCTGC AAGGCGATTA
 5351 AGTTGGGTAA CGCCAGGGTT TTCCAGTCA CGACGTTGTA AAACGACGGC
 5401 CAGTGAATTA GTACTCTAGC TTAAGTAAGC CATTTTGCAA GGCATGGAAA
 5451 AATACATAAC TGAGAATAGA GAAGTTCAGA TCAAGGTTAG GAACAGAGAG
 5501 ACAGGAGAAT ATGGGCCAAA CAGGATATCT GTGGTAAGCA GTTCCTGCCC
 5551 CGGCTCAGGG CCAAGAACAG TTGGAACAGC AGAATATGGG CCAAACAGGA
 5601 TATCTGTGGT AAGCAGTTCC TGCCCCGGCT CAGGGCCAAG AACAGATGGT
 5651 CCCCAGATGC GGTCCCGCCC TCAGCAGTTT CTAGAGAACC ATCAGATGTT
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 6101 AATGTTTGCG CCTGCGTCTG TACTAGTTGG CTAAGTAGAT CTGTATCTGG
 6151 CGGTCCCGCG GAAGAACTGA CGAGTTCGTA TTCCCGGCCG CAGCCCCCTG
 6201 GAGACGTCCC AGCGGCCTCG GGGGCCCGTT TTGTGGCCCA TTCTGTATCA
 6251 GTTAACCTAC CCGAGTCGGA CTTTTTGGAG CTCCGCCACT GTCCGAGGGG
 6301 TACGTGGCTT TGTTGGGGGA CGAGAGACAG AGACACTTCC CGCCCCCGTC
 6351 TGAATTTTTG CTTTCGGTTT TACGCCGAAA CCGCGCCGCG CGTCTTGTCT
 6401 GCTGC

pMP71-CD3ζ-2A-CD3ε-2A-CD3δ-2A-CD3γ-IRES-GFP

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Sequence of F5-TCR β chain insert

GCGAGCAGGCCCTAAGCTTGGATCCGAATTCTTACTTGTACAGTCGACTCATCAGCTG
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CCATAGCGG

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